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(54) Title: A CELL–FREE SYSTEM OF MITOCHONDRIA–DEPENDENT APOPTOSIS, AND METHODS OF USE THEREOF

(57) Abstract

In accordance with the present invention, there are provided cell–free methods for identifying compounds that modulate mitochondria–dependent apoptosis. Also provided are methods for identifying compounds that modulate a specific mitochondria–dependent–stage of apoptosis.

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A CELL-FREE SYSTEM OF MITOCHONDRIA-DEPENDENT APOPTOSIS, AND METHODS OF USE THEREOF

This application claims the benefit of priority of copending provisional application Serial No. 60/054,643, filed August 4, 1997, now pending.

This invention was funded in part by NIH Grant Nos. AG12282 and CA69381. Accordingly, the United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

Apoptosis is the term used to describe a type of cellular death that occurs in many tissues as a normal physiological process. Also, referred to as "programmed cell death," this form of cellular demise involves the activation in cells of a built-in genetic program for cell suicide wherein cells essentially autodigest. The remnants of these dead cells are then cleared almost without a trace by neighboring phagocytic cells, without resulting in inflammation or scarring. Apoptosis thus stands in marked contrast to cell death caused, for example, by oxygen-deprivation in the settings of myocardial infarction or stroke, where cells lose their energy supplies, rupture, and spill their contents into the extracellular milieu.

Accumulating evidence suggests that neuronal apoptosis plays a role not only in nervous system development, but also in neurological disease states (Bredesen, Apoptosis II: the molecular basis of apoptosis in disease, Cold Spring Harbor Laboratory Press, 397-421 (1994); Bredesen, Ann. Neurol 38:839-851 (1995); Thompson, Science 267:1456-1462 (1995). Evidence has been presented for apoptotic neuronal cell death in stroke (Linnik *et al.*, Stroke 24:2002-2009 (1993)), Parkinson's disease (Mochizuki *et al.*, J. Neurol. Sci.

137:120-123 (1996)), Alzheimer's disease (Cotman and Anderson Mol. Neurobiol. 10:19-45 (1995); Dragunow *et al.*, Neuroreport 6:1053-1057 (1995); Smale *et al.*, Exp. Neurol. 133:225-230 (1995); La Ferla *et al.*, Nat. Genet. 9:21-30 (1996); Su *et al.*, Neuroreport 5:2529-2533 (1994)), amyotrophic lateral sclerosis (ALS) (Yoshiyama *et al.*, Acta Neuropathol (Berl) 88:207-211, (1994)), human immunodeficiency virus encephalopathy (Gelbard *et al.*, Neuropathol. Appl. Neurobiol. 21:208-217, (1995); Petito *et al.*, Am. J. Pathol.,146:1121-1130 (1995)), cerebral trauma (Rink *et al.*, Amer. J. Path. 147:1-9, (1995)); Ferrer *et al.*, Brain Path. 4:115-122 (1994)); Huntington's disease (Yoshiyama *et al.*, supra, (1994)); Thomas *et al.*, Exp. Neurol. 133: 265-272, (1995); Dragunow *et al.*, Exp. Neurol., 133:265-272, (1995)), and other diseases (Bredesen, supra, (1995)). Furthermore, mutations in the anti-apoptotic gene NAIP (neuronal apoptosis-inhibitory protein) are associated with spinal muscular atrophy (Roy *et al.*, Cell 80:167-178 (1995)).

In addition to evidence of neuronal apoptosis in disease states, some of the mutations associated with neurodegenerative diseases have been shown to have pro-apoptotic effects in cell culture models. For example, mutations of sod1, which encodes copper/zinc superoxide dismutase (CuZnSOD), are associated with familial amyotrophic lateral sclerosis (Rosen *et al.*, 362:59-62, (1993)), and these mutations have been shown to convert CuZnSOD from manifesting an anti-apoptotic effect on cultured neural cells to manifesting a pro-apoptotic effect (Rabizadeh *et al.*, Proc. Natl. Acad. Sci. USA, 92:3024-3028 (1995). Similarly, Alzheimer's disease-associated mutations at amyloid precursor protein residue 717 (APP717 mutations) have been shown to be pro-

apoptotic in a subclone of cos cells (Yamatsuji *et al.*, EMBO J., 15:498-509, (1996); presenilin-2 mutants enhance the pro-apoptotic effect of presenilin-2 (Wolozin *et al.*, Science 274:1710-1713 (1996)); and the Machado-Joseph disease triplet repeat expansion confers a pro-apoptotic phenotype in cell culture (Ikeda *et al.*, Nat. Genet., 13:196-202, (1996).

A variety of whole-cell apoptotic assays have been developed in recent years. In addition, cell-free systems have proven to be invaluable for the study of a number of cellular events, such as mitosis (Lee *et al.*, Proc. Natl. Acad. Sci. USA, 93:352-356, (1996)), protein translocation and post-translational modification (Rothman, Harvey Lect., 86:65-85 (1990), as well as apoptosis of non-neural cells (Lazebnik *et al.*, Proc. Natl. Acad. Sci. USA, 92:9042-9046 (1993); Newmeyer *et al.*, Cell 79:353-364 (1994); Enari *et al.*, EMBO J. 14:5201-5208 (1995); Martin *et al.*, J. Biol. Chem., 270:6425-6428, (1995a), Martin, S.J., EMBO J., 14:5191-5200 (1995b), The EMBO J., 15:2407-16 (1996); Liu *et al.*, Cell 86:147-157 (1996)), among others. However, neuronal-based cell-free apoptotic assays are lacking.

In addition, it would be desirable to have bioassays that distinguish a particular test-compounds activity between mitochondria-dependent, pre-mitochondria-dependent and post-mitochondria-dependent stages of apoptosis. It would also be desirable to identify compounds that modulate mitochondria-dependent apoptosis. The present invention satisfies this need and provides related advantages as well.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided cell-free methods for identifying compounds that modulate mitochondria-dependent apoptosis. Also provided are bioassays that can distinguish a particular test-compounds activity between mitochondria-dependent, pre-mitochondria-dependent and post-mitochondria-dependent stages of apoptosis.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows staurosporine and tamoxifen-activated neural apoptosis. (**A**) Percent apoptotic cells versus time in hours. CSM-25 cells were incubated at 34°C with 10 μM staurosporine (white rectangles), or 100 μM tamoxifen (black rectangles) for the indicated times. Apoptotic cells were judged morphologically. (**B**) Proteolytic profile of protein substrates selectively cleaved during staurosporine-initiated neural apoptosis. CSM-25 cells were incubated at 34°C with 10 μM staurosporine for the indicated times. Cell lysates were made at the indicated time points, and subjected to Western blot analysis.

Figure 2 shows that tamoxifen-primed extract activates neural cell-free apoptosis. (**A**) Nuclear morphological changes in CSM nuclei incubated between 0 and 2 hours at 34°C in a 16,000 g extract made from tamoxifen-primed NSC-19 cells. (**B**) Percent apoptotic nuclei incubated as in (A) in either normal or primed extract. (**C**) Agarose gel electrophoresis of internucleosomal DNA fragmentation of rat liver nuclei incubated for 2 hours at 34°C in a 16,000 g extract made from tamoxifen-primed CSM-25 cells. (**D**) Selective proteolytic cleavage of key substrates from a cell-free

reaction of HeLa nuclei incubated for the indicated times at 37°C in a 16,000 g extract made from tamoxifen-primed CSM-25 cells. Cleavage was prevented by 1 μM Ac-DEVD-CHO, but not prevented by 1 μM Ac-YVAD-CHO. (**E**) The activity of CPP32-like caspases as measured by DEVD-pNA hydrolysis. The CPP32-like caspase activity of a tamoxifen-primed NSC-19 extract after a 2 hour incubation at 37°C in is given by the *upper* line. The DEVD activity of a normal NSC-19 extract, and the YVAD activity of a tamoxifen-primed NSC-19 extract fall at or below the *lower* line.

Figure 3 shows that atractyloside activates neural cell-free apoptosis. Atractyloside (5 mM) was incubated in a 3,000 g extract made from CSM-25 cells for 1.5 h at 37°C. The activation of apoptosis was measured by fodrin cleavage. Atractyloside also induced cell-free apoptosis in a system composed of rat liver mitochondria and 16,000 g extract from CSM-25 cells. However, atractyloside incubated in a 16,000 g extract alone did not lead to cell-free apoptosis.

Figure 4 shows that mastoparan activates neural apoptosis. Mastoparan induces apoptosis in cultured rat cerebellar neuron precursors (the R2 cell line) as measured by cell death using propidium iodide staining of DNA in cells with a compromised plasma membrane (see Rabizadeh *et al.*, Proc. Natl. Acad. Sci. USA 92:3024-3028 (1993)).

Figure 5 shows that mastoparan activates neural and neuronal cell-free apoptosis. (**A**) Fodrin cleavage and CPP32 processing in a neural cell-free system composed of a 3,000 g extract (containing mitochondria) made from CSM-25 cells, in a neural cell-free system composed of mouse liver mitochondria in a 16,000 g

extract from NT2 cells, and in a neuronal cell-free system composed of rat neuronal mitochondria in a 16,000 g extract from primary cerebellar neurons. All systems were incubated for 1.5 h at 37°C with 100 μM mastoparan. Mastoparan did not prime a 16,000 g extract without mitochondria. **(B)** Mastoparan induced release of cytochrome c from mitochondria. Mastoparan (100 *m*M) incubated for 1h at 37°C with mouse liver mitochondria led to the release of cytochrome c, as measured by Western blot of the supernatant from the mitochondrial pellet. **(C)** The processing of DEVD-pNA substrate at 37°C by a 16,000 g normal NT2 extract activated by the concentrated supernatant from (B), is shown in the *upper* curve. Note the characteristic ~500 second activation lag time. The activity of a 16,000 g normal NT2 extract, of a 16,000 g normal NT2 extract incubated with mastoparan, and mastoparan in buffer was less than or equal to the activity shown by the *lower* curve.

Figure 6 shows that cytochrome c and dATP activate neural and neuronal cell-free apoptosis. **(A)** Nuclear fragmentation of HeLa nuclei incubated between 1 and 1.5 h at 37°C in a 16,000 g NT2 extract with 10 μM cytochrome c and 1 mM dATP. **(B)** DNA fragmentation of CSM nuclei incubated for 1h at 37°C in a 16,000 g CSM extract with 10 μM cytochrome c and 1 mM dATP. **(C)** Proteolysis of fodrin and the processing of CPP32 in extracts incubated for 1h at 37°C with 1 mM dATP, and various forms of cytochrome c (10 μM). While horse heart cytochrome c activated both 16,000 g NT2 extracts, and 16,000 g extracts from rat primary cerebellar neurons, yeast and acetylated horse cytochrome c did not activate extracts. A 16,000 g CSM extract made from Bcl-2 overexpressing cells, with or without mitochondria from Bcl-2 overexpressing cells, and a 3,000 g extract from such cells is activated by cytochrome c/dATP. A 30 min

pre-incubation of 16,000 g extract at 37°C renders the extract incapable of activation by cytochrome c/dATP. Furthermore, the peptide inhibitor zVAD-fmk prevents the activation. (D) Activation of CPP32-like capase in a 16,000 g NSC-34 extract incubated 37°C with 1 mM dATP and 10 μM cytochrome c, shown by the *upper* curve, as measured by hydrolysis of DEVD-pNA. The activity of extract alone is shown by the *lower* curve. The activities of yeast and partially acetylated cytochrome c in the above system, and cytochrome c/dATP in buffer, lie at or below the activity of normal extract.

Figure 7 shows that CPP32 processing in mitochondria-dependent activation of cell-free apoptosis. Here we illustrate the processing of CPP32 in the neural cell-free system for tamoxifen, mastoparan, and cytochrome c/dATP. Tamoxifen (Tam) induces apoptosis at the pre-mitochondrial level (cells), but does *not* induce apoptosis at the mitochondrial level (mitochondria and extract), or the post-mitochondrial level (extract). Similar results were obtained for staurosporine. Mastoparan (Mast) induces apoptosis at the pre-mitochondrial level (cells) and at the mitochondrial level (mitochondria and extract), but does *not* induce apoptosis at the post-mitochondrial level (extract). Similar results were obtained for atractyloside. Cytochrome c/dATP (Cytc) induces apoptosis at the mitochondrial level (mitochondria and extract) and at the post-mitochondrial level (extract), but does *not* induce apoptosis at the pre-mitochondrial level (cells).

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided cell-free methods for identifying compounds that inhibit mitochondria-dependent apoptosis, comprising:

a) providing a non-activated cell extract, wherein said cell extract further comprises mitochondria, and a test-compound;

b) contacting said cell extract with a mitochondria-dependent apoptosis-inducing agent; and

c) identifying a compound that inhibits apoptosis. In a preferred embodiment of each of the invention methods described herein, the cell extracts employed are neuronal cell extracts.

As used herein, the phrase "cell-free" refers to a system that does not include whole, intact cells. The phrase "mitochondria-dependent apoptosis" refers to the occurrence of cell-free apoptosis only when there are mitochondria present in the system, such as in cell-free extracts having mitochondria added therein, whereas cell-free apoptosis does not occur in the absence of mitochondria.

The phrase "non-activated cell extract" refers to a normal cytosolic cell extract obtained from a cell that has not been primed or otherwise induced to undergo apoptosis. It has been found that a non-activated cell extract in combination with mitochondria added therein is particularly advantagous in bioassays for identifying compounds that modulate mitochondria-dependent apoptosis. For use herein, the cell extracts are preferably obtained from neuronal cells.

The non-activated cell extracts employed herein are preferably prepared so that the pelleted mitochondria

is not disrupted such that cytochrome c, or any other contaminating priming agent, is released into the cytosol causing the extract to self-prime. Preferably, the non-activated cell extract is prepared by centrifuging cell lysates at about 16,000 g for about 10-30 minutes, as described in Example 1.C. Thus, in a particular embodiment of the present invention, the cell extracts are substantially free of whole cells, organelles, and membrane fractions that pellet at about 16,000g for 10-30 minutes, such as nuclei, mitochondria, lysosomes, and the like. In this particular embodiment, when mitochondria are added to the non-activated cell extracts the invention cell-free assay system is substantially free of non-mitochondria membrane organelles that are greater than or equal to the weight of mitochondria, which are typically present in the cytosol.

Accordingly, as used herein, the phrase "substantially free of non-mitochondria membrane organelles" refers to cell extracts that do not include, for example, nuclei, mitochondria, lysosomes, and any contaminating inducing agents (such as cytochrome c, AIF, and the like) that might cause the cell extract to self-prime. Cell extracts that are substantially free of non-mitochondria membrane organelles can be prepared as described, e.g., in Example I.C.

The term "neuronal," as used herein in the context of cell-types, refers to primary mammalian neuronal cells and mammalian neural cell-lines, and the like. Neuronal cells suitable for use in the invention methods include, for example, primary cultures of mammalian cerebellar neurons, the human teratocarcinoma-derived neuronal precursor cell line NT2/D1 (Pleasure and Lee, J. Neurosci. Res. *35*, 585-602 (1993)), the mouse motor neuron-like cell lines NSC-34 and NSC-19 (Cashman, Devel. Dyn. *194*, 209-221. (1992)), and the rat central nervous

system (CNS) neuron-like cell line CSM-25 (Durand *et al.*, Soc Neurosci Abs *16*, 40 (1990); Zhong *et al.*, Mol. Brain Res. *19*, 353-355 (1993); Zhong *et al.*, Proc. Natl. Acad. Sci. USA *90*, 4533-4537 (1993)) and the like. The term "mammalian" refers to the variety of species from which neuronal cells for use in the invention methods can be obtained, e.g., human, rat, mouse, rabbit, monkey, baboon, bovine, porcine, ovine, canine, feline, and the like.

Mitochondria employed in the invention methods can be obtained from either the same or different species as the cell extracts. Likewise the mitochondria can be obtained from either the same or differenct cell-types as the cell extracts. Methods for isolating mitochondria are described, for example, in Example I.F., and are otherwise well-known in the art (see, e.g., Hovius *et al.*, Biochem. Biophys. Acta, 1021:217-226 (1990); Moreadith and Fiskum, Anal. Biochem. 137:360-367 (1984); and the like).

As used herein, the term "test-compound" refers to a compound to be tested in the invention bioassays. The test-compounds can be obtained from diverse variety of compound libraries that are generally available to those of skill in the art. Single compounds can thus be identified and selected from the test-compounds subjected to the invention bioassays as "compounds" having the desired biological activity.

As used herein, the phrase "mitochondria-dependent apoptosis-inducing agent" refers to agents that induce apoptosis when, preferably only when, there is mitochondria present in the system, such as in whole cells or in a cell-free extracts having mitochondria therein. Suitable agents include, for example, those

compounds that can disrupt the inner and/or outer membrane of the mitochondria, such as by forming pores in, or by lysing, the membrane. Such agents can include proteins or peptides having "mitochondrial presequences" that are rich in basic amino acids, adopt an α-helical conformation, and result in an amphipathic structure (see, e.g., Nicolay et al., J. Bioeng. and Biomembranes, 26:327-334 (1994); Baker et al., Nature, 349:205-208 (1991); and Pfanner et al., Trends Biochem. Sci., 16:63-67(1991)). An exemplary mitochondrial presequence is the 25-residue presequence of cytochrome c-oxidase subunit IV (Maduke et al., Science, 260:364-367 (1993)).

Other exemplary mitochondria-dependent apoptosis-inducing agents include mastoparan, atractyloside, cardiolipin (see, e.g., Hovius et al., FEBS Lett., 330:71-76 (1993); Hovius et al., Biochim. Biophys. Acta, 1021:217-226 (1990)), adrenodoxin precursor (see, e.g., Ou et al., J. Biochem. 103:589-595 (1988)), synthetic mitochondrial presequences (see, e.g., Roise, PNAS, USA, 89:608-612 (1992); and Furuya et al., EMBO J., 10:1759-1766 (1991)), and the like. A preferred mitochondria-dependent apoptosis-inducing agent, for use herein, is mastoparan.

In addition, the invention methods described herein can be used to identify additional mitochondria-dependent apoptosis-inducing agents. For example, compounds can be identified that induce apoptosis in non-activated cell extracts only in the presence of mitochondria. Such compounds can be identified using an invention cell-free method for identifying compounds that activate mitochondria-dependent neuronal apoptosis described hereinafter.

As used herein, the phrase "apoptosis" refers to the well-known process of programmed cell death. There is a variety of well-known, generally accepted *in vitro* indicia of apoptosis, including nuclear morphological changes, internucleosomal fragmentation of DNA, the selective proteolysis of substrates, and the activation of CPP32-like caspases. For example, the substrates of the caspase family of cysteine proteases have received considerable attention because cleavage of these substrates offers molecular mechanisms for many of the hallmark morphological and functional changes exhibited by apoptotic cells (Casiano *et al.*, J. Exp. Med., 184:765-770, (1996). For example, the cleavage of fodrin leads to morphological alterations such as process retraction, cellular rounding, and bleb formation. The cleavage of nuclear substrates such as the lamins, NuMA, and topoisomerase I, is believed to be associated with the dissolution of the nuclear membrane, chromatin condensation, and nuclear fragmentation. Furthermore, the cleavage of substrates during apoptosis can lead to activation, not just inactivation. For example, while PARP cleavage has been reported to lead to inactivation, Lazebnik *et al.*, Nature, 371:346-347, (1994), the cleavage of PKC-δ results in the activation of the enzyme (Emoto *et al.*, The EMBO J., 24:6148-6156, (1995).

Thus, the occurrence of apoptosis in cell-free systems, such as the invention non-activated neuronal extract having mitochodria therein, can be assessed by detecting the relative levels of: caspase processing (i.e., the cleavage of the pro-caspase to active forms; see, e.g., Casciola-Rosen et al., 1996, J. Exp. Med.,183:1957-1964; Tewari et al, 1995, J. Biol. Chem., 32:18738-18741; Tewari et al, 1995, Cell, 81:801-809), caspase activation, cytosolic substrate cleavage, the release of cytochrome c from mitochondria, and the like.

Exemplary cytosolic substrates that are cleaved as a result of apoptosis are set forth in Table 1, and include: fodrin, CPP32, PKC-δ, and the like.

When nuclei is optionally included in the cell-free system, the occurrence of apoptosis can be assessed by, in addition to the methods described above, detecting: chromatin condensation, shrinkage and fragmentation of the nuclei, and the like (see, for example, Zanzami et al., J. Exp. Med., 183:1533-1544 (1995); Newmeyer et al., Cell, 79:353-364 (1994)). In addition, nuclear substrates that are cleaved as a result of apoptosis are also set forth in Table 1, and include: DNA topoisomerase (Liu, Ann. Rev. Biohem. *58*, 351-375 1989), lamin B (Lazebnik et al., Proc. Natl. Acad. Sci. USA *92*, 9042-9046. 1995), NuMA (Compton, 1994), PARP (Lazebnik et al., Supra 1994), and U1-70kDa (Casciola-Rosen et al., J. Biol. Chem. *49*, 30757-30760. 1994), and the like.

As used herein, the terms "modulates", "modulation", or grammatical variations thereof, in the context of modulating apoptosis refer to either the inhibition (such as with antagonists) or activation (such as with agonist) of apoptosis. The modulation of apoptosis at the mitochondria-dependent stage can be determined, for example, by methods described herein, such as in Example 3, or the like.

In accordance with another embodiment of the present invention, there are provided cell-free methods for identifying a compound that activates mitochondria-dependent apoptosis, said method comprising:

a) providing a non-activated cell extract in the presence and absence of mitochondria;

b) contacting said cell extract with a test-compound; and

c) identifying a compound that activates mitochondria-dependent apoptosis. A compound is identified as a mitochondria-dependent activator of apoptosis if, for example, it activates apoptosis in the presence of mitochondria, and does not activate apoptosis in the absence of mitochondria. In a preferred embodiment of this invention method, the cell extract is obtained from neuronal cells.

In yet another embodiment of the present invention, there are provided cell-free methods for identifying a compound that modulates neuronal apoptosis, said method comprising:

a) providing a neuronal cell extract containing mitochondria,

b) contacting said cell extract with a test-compound, and

c) identifying a compound that modulates apoptosis.

In one particular aspect of this invention method, the modulating compound identified inhibits apoptosis and the extract further comprises a mitochondria-dependent apoptosis inducing agent. In another aspect of this invention method, the modulating compounds identified are mitochondria-dependent, as such compounds activate apoptosis only in the presence of mitochondria and do not activate apoptosis in the absence of mitochondria.

In accordance with another embodiment of the present invention, there are provided cell-free methods for identifying compounds that inhibit mitochondria-dependent neuronal apoptosis, the methods comprising:

a) providing a non-activated neuronal cell extract in the presence and absence of a test-compound, wherein said cell extract further comprises mitochondria, and a mitochondria-dependent apoptosis-inducing agent; and

b) identifying a compound that inhibits apoptosis. A compound is identified as an inhibitor of apoptosis if, for example, it decreases the levels of caspase processing or substrate cleavage when the test-compound is present compared to such levels in the absence of test-compound.

As set forth in Examples 2-4, cell-free apoptosis is activated in a pre-mitochondria-dependent, mitochondria-dependent, and post-mitochondria-dependent stages, by tamoxifen, mastoparan, and cytochrome c, respectively, allowing a functional ordering of these pro-apoptotic modulators. Thus, in accordance with another embodiment of the present invention, there are provided methods for identifying compounds that modulate apoptosis specifically at a mitochondria-dependent apoptotic stage, pre-mitochondrial-dependent apoptotic stage, or post-mitochondria-dependent stage. For example, an invention method for identifying a compound that specifically modulates mitochondria-dependent apoptosis comprises:

(1) determining whether a test-compound modulates mitochondria-dependent apoptosis;

(2) determining whether said test-compound modulates pre-mitochondria-dependent apoptosis;

(3) determining whether said test-compound modulates post-mitochondria-dependent apoptosis; and

(4) identifying a compound that modulates mitochondria-dependent apoptosis.

As used herein, the phrase "pre-mitochondria-dependent apoptosis" refers to the stage of apoptotic induction that requires components other than mitochondria, such as contained in whole cells. As set forth in Example 2, staurosporine and tamoxifen induce apoptosis in whole cells from which active extracts may then be prepared, but do not induce apoptosis directly in cell extracts. This indicates that induction of apoptosis by these agents requires an intact signaling mechanism that is absent in the invention cell-free systems described herein. Thus, also contemplated herein are neuronal cell-free apoptotic systems, that include additional purified fractions such as plasma membranes (Meier *et al.*, J. Cell Biol. 998:991-1000 (1984) and lysosomes (Ohshita and Kido, Anal. Biochem. 230:41-47 (1995)). Since this type of cell-free system could represent a more upstream system, agents like tamoxifen might then induce apoptosis without the need for intact cells.

As used herein, the phrase "mitochondria-dependent apoptosis" refers to apoptotic induction that requires the presence of mitochondria. As set forth in Example 3, unlike staurosporine and tamoxifen, atractyloside and mastoparan, both of which induce the mitochondrial inner membrane permeability transition (Zamzami *et al.*, J. Exp. Med. 183:1533-1544 (1996); Pfeiffer *et al.*, J. Biol. Chem., 270:4923-4932 (1995)), activate neural cell extracts directly, without the requirement for whole cells, but with the requirement for mitochondria. This lack of need for intact cells indicates that the points of apoptosis activation by staurosporine and tamoxifen lie upstream from that of atractyloside and mastoparan. Thus, it is contemplated herein that mastoparan induces mitochondria to release an apoptotic activator such as cytochrome c.

As used herein, the phrase "post-mitochondria-dependent apoptosis" refers to cell-free apoptotic induction that does not require the presence of mitochondria. As set forth in Example 4, it has been demonstrated that cytochrome c and dATP, added together, activate neural cell extracts in a manner that is independent of mitochondria. These results indicate that cytochrome c and dATP induce apoptosis at a point distal to those of staurosporine, tamoxifen, atractyloside, and mastoparan. With respect to cytochrome c/dATP activation, it should be noted that not all forms of cytochrome c activate the system. Yeast (ISO-1) and partially acetylated horse cytochrome c are incapable of activating the system. In yeast, lysine 72 is trimethylated (Brayer and Murphy, Scott, R.A. and Mauk, A.G. eds. (University Science Books), Chapter 3, pp 107. (1996). Furthermore, the acetylation of lysines, Azi *et al.*, Biochem. Biophys. Res. Commun., 65:597-603 (1975)) prevents horse cytochrome c from activating the system. Thus, whether acetylated or methylated, lysine 72 is a good candidate as a residue required for cytochrome c to activate cell-free apoptosis.

In addition, pre-incubation of an extract for 30 min at 37°C renders it insensitive to cytochrome c activation, implying a temperature sensitive component in the extract is required for cytochrome c/dATP activation. This finding is similar to that of Susin *et al.* Supra (1996) involving AIF. In addition, Bcl-2 produces an anti-apoptotic effect at the pre-mitochondria-dependent and mitochondria-dependent stages, but cannot protect against the post-mitochondria-dependent activation of apoptosis by cytochrome c.

As used herein, the phrase "specifically modulates mitochondria-dependent apoptosis" refers to a

compound that modulates only mitochondria-dependent apoptosis and does not modulate pre- or post-mitochondria-dependent apoptosis. Methods for determining whether a test-compound activates mitochondria-dependent apoptosis are described, for example, in Example 3. As set forth in Example 3, it has been found that mastoparan, a peptide toxin from wasp (*Vespula lewisii*) venom (Hirai *et al.*, Chem. Pharm. Bull. (Tokyo) 27:1942-4 (1979)), induces apoptosis in a normal cell extract, provided that mitochondria are added to the extract, but does not induce apoptosis in the absence of mitochondria. This is the first report of mitochondria-dependent activation of cell-free apoptosis in a cell extract.

Methods for determining whether a test-compound activates pre-mitochondria-dependent apoptosis are described in, e.g., Example 2. As set forth in Example 2, it has been found that tamoxifen, an anti-oestrogenic and anti-neoplastic agent (Fenwick *et al.*, Br. J. Pharmacol. 59:191-199 (1977); Pollak, Digestion 57 Suppl., 1:29-33 (1996), activates apoptosis in whole cells, from which an apoptotically active extract may then be prepared. In addition, the kinase inhibitor staurosporine (Koh *et al.*, Exp. Neurol. 135:153-159 (1995)) was found to induce neuronal apoptosis in whole cells. However, neither tamoxifen or staurosporine activates apoptosis in a normal cell extract, whether or not mitochondria are added to the extract. Thus, test-compounds that produce the same results as tamoxifen or staurosporine can readily be identified by the methods of, e.g., Example 2 as pre-mitochondria-dependent apoptosis activating compounds.

Methods for determining whether a test-compound activates post-mitochondria-dependent apoptosis are

described, e.g., in Example 4. As set forth in Example 4, it has been found that cytochrome c and dATP, added together, do not activate apoptosis in whole cells. However, they do activate a normal cell extract, whether or not mitochondria are added to the extract. Furthermore, they activate a normal extract made from *bcl-2* overexpressing cells, whether or not mitochondria from *bcl-2* overexpressing cells are present. Thus, those of skill in the art, employing the methods of Example 4, can readily identify test-compounds that reproduce the results obtained by cytochrome c and dATP as compounds that activate post-mitochondria-dependent apoptosis.

Taken together, the data provided herein teach a general temporal ordering of neuronal apoptotic events as pre-mitochondria-dependent, mitochondria-dependent, and post-mitochondria-dependent. Thus, methods are provided herein for identifying compounds that modulate mitochondria-dependent apoptosis. Such methods are useful, for example, for early high-throughput screening of random test-compounds to reduce the number of test-compounds selected for further research, when it is desired to obtain compounds that exhibit apoptotic activity or lack such activity at a particular mitochondria-dependent-stage of apoptosis. In addition, compounds known to modulate apoptosis can be subjected to the invention methods to identify the specific apoptotic stage of such activity.

In addition, those of skill in the art recognize that in lieu of identifying a compound that specifically modulates mitochondria-dependent apoptosis, one can select a compound that specifically modulates either pre-mitochondria-dependent apoptosis, or post-mitochondria-dependent apoptosis, or any combination thereof. Thus, also contemplated herein are methods for identifying a compound that modulates a specific

mitochondria-dependent-stage of apoptosis, said method comprising:

(1) determining whether a test-compound modulates mitochondria-dependent apoptosis;

(2) determining whether said test-compound modulates pre-mitochondria-dependent apoptosis;

(3) determining whether said test-compound modulates post-mitochondria-dependent apoptosis; and

(4) identifying a compound that modulates a specific mitochondria-dependent-stage of apoptosis.

As used herein, the phrase "mitochondria-dependent-stage of apoptosis" refers to one or more of pre-mitochondria-dependent apoptosis, mitochondria-dependent apoptosis, or post-mitochondria-dependent apoptosis, as described above. For example, step (4) above, can be identifying a compound that modulates mitochondria-dependent apoptosis, wherein the compound does not modulate pre-mitochondria-dependent or post-mitochondria-dependent apoptosis. Alternatively, step (4) above, can be identifying a compound that modulates pre-mitochondria-dependent apoptosis, wherein the compound does not modulate mitochondria-dependent or post-mitochondria-dependent apoptosis. In addition, step (4) above, can be identifying a compound that modulates post-mitochondria-dependent apoptosis, wherein the compound does not modulate pre-mitochondria-dependent or mitochondria-dependent apoptosis.

All U.S. patents and all publications mentioned herein are incorporated in their entirety by reference thereto. The invention will now be described in greater detail by reference to the following non-limiting examples.

Example 1
General Methods

Unless otherwise stated, the present invention was performed using standard procedures as described, for example, in Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1982); Sambrook et al., Molecular Cloning: A Laboratory Manual (2 ed.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA (1989); Davis et al., Basic Methods in Molecular Biology, Elsevier Science Publishing, Inc., New York, USA (1986); or Methods in Enzymology: Guide to Molecular Cloning Techniques Vol.152, S. L. Berger and A. R. Kimmerl Eds., Academic Press Inc., San Diego, USA (1987).

The general methods described in Example 1 were employed to carry out Examples 1-5 described herein.

A. Cell Cultures.

Primary cultures of *rat cerebellar neurons* were prepared by a procedure that combines elements of the method of Schousboe *et al.*, Alan R. Liss, Inc., 203-206 (1989), with that of Cole and deVellis, Alan R. Liss, Inc., 121-133 (1989), with some modifications. Sprague-Dawley rat pups between 5 - 7 days were alcohol sterilized and killed by decapitation. Gross dissection of each brain, and micro-dissection of the cerebellum, was performed as per Cole and deVellis. The dissection medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 4.5 g/l glucose and L-glutamine (Mediatech cellgro). The Nitex bag method of Lu *et al.*, J. Neurosci. Res., 5:447-463 (1980) (see also Cole and de Vellis, Supra), was used to dissociate the cerebellar tissue, with notable exceptions. Instead of a Nitex

monofilament bag, a double-layer fiberglass mesh bag (18 openings per linear inch) of the same dimensions was used. After the bag was sterilized with ethanol, the cerebellar tissue was inserted into the top of the bag, and gently teased through the mesh with very light strokes of a glass rod into the same medium as used for the dissection, only now containing 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (Sigma Chemical Company) and 1% Penicillin-Streptomycin (10,000 units/ml penicillin G sodium and 10.0 μg/ml streptomycin sulfate; GibcoBRL). The cell/media suspension was then poured through a sieve with 25 openings per linear inch, and then poured through a sieve with 45 openings per linear inch. Next, the cells were plated at a density of about 10^6/ml on ~2000 mm^2 tissue culture plates (Falcon Integrid).

All the other adherent cell lines grown herein, es. CSM, NSC, NT2, Hela were also cultured on these plates. For the cerebellar neurons, the plates were coated with poly-L-lysine (Sigma) at 20 μg/ml, incubated at 37°C for 4 h, rinsed once with sterile water, and twice with phosphate buffered saline (PBS). Twenty to thirty pups yield about two such plates of cerebellar neurons. The cells were incubated at 37°C, with a 5% CO_2 / 95% air mixture. After a 48 h incubation, the cells were treated with cytosine β-D-arabinoside (Sigma) at 40 mM (final concentration) to eliminate glia, and any cerebellar neurons still capable of undergoing mitosis. Four days later, only terminally differentiated cerebellar neurons remained. Cytoplasmic extract was made a day later.

CSM 14.1 cells (Durand *et al.*, Soc Neurosci Abs, 16:40 (1990); Zhong *et al.*, Mol Brian Res., 19:353-355 (1993a); Proc. Natl. Acad.Sci. USA, 90:4533-4537

(1993b) were made using E14 rat mesencephalon primary cultures, and immortalized by using the pSVtsA58 retrovirus. This line is neural by neurofilament staining (NF-H) at the restrictive temperature 39°C, and by expression of tyrosine hydroxylase mRNA, suggesting that it is a dopaminergic precursor. CSM-25, a subclone of CSM 14.1 was selected for its high propensity to undergo apoptosis following serum withdrawal. CSM-25 cells were grown at 34°C, with a 5% CO_2/95% air mixture, in the same medium as that used for the cerebellar neurons.

The NSC-34 and NSC-19 cell lines mimic selected aspects of motor neuron development in an immortalized clonal system. These cell lines are mouse-mouse neural hybrids, developed by fusing the aminopterin-sensitive neuroblastoma N18TG2 with motor neuron-enriched embryonic day 12 - 14 spinal cord cells (Cashman *et al.*, Devel. Dyn. 194:209-221 (1992) incorporated herein by reference in its entirety). The NSC-34 and NSC-19 hybrids display a mutipolar neuron-like phenotype, express choline acetyltransferase, and induce twitching in cocultured mouse myotubes. Beyond this, they express the additional properties expected of motor neurons, including generation of action potentials, expression of neurofilament triplet proteins, and acetylcholine synthesis, storage, and release. Furthermore, NSC-34 cells induce acetylcholine receptor clusters on cocultured myotubes, and undergo a vimentin-neuro-filament switch with maturation in culture, similar to that occurring in neuronal development. NSC-34 and NSC-19 cells were grown at 37°C, with a 5% CO_2 / 95% air mixture, in Dulbecco's modified Eagle's medium (DMEM) (GibcoBRL), supplemented with 4.5 g/l glucose and L-glutamine (Mediatech cellgro) and containing 10% heat-inactivated (56° for 30 min) fetal bovine serum (FBS)

(Summit Biotechnology, Ft. Collins Col.) and the penicillin/streptomycin formulation given above.

The human teratocarcinoma cell line NT2/D1 can be manipulated following treatment of retinoic acid to yield > 99% pure cultures of terminally differentiated NT2-N neurons (Pleasure and Lee, J. Neurosci. Res. 35:585-602 (1993)). The NT2/D1 neuronal precursor line is a stem cell line that cannot be induced to yield derivatives other than neurons. This is in sharp contrast to other teratocarcinoma cell lines which yield multiple cell types from multiple germ cell layers. NT2/D1 cells were grown at 37°C, with a 5% CO_2 / 95% air mixture, in Opti-MEM supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (Sigma Chemical Company) and containing the penicillin/streptomycin formulation given above.

The R2 cell line is a conditionally immortalized cerebellar neural line (Rabizadeh *et al.*, J. Neurochem. 61:2318-2321 (1993); Greenberg *et al.*, Proc. Natl. Acad. Sci. USA, 81:969-973 (1984)). The R2 cells were grown at 34°C in the same medium as that used for the cerebellar neurons.

Jurkat and Hela cells were grown at 37°C, with a 5% CO_2 / 95% air mixture in RPMI 1640 medium supplemented with L-glutamine (Mediatech cellgro) and containing 10% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS) (Sigma Chemical Company) and the penicillin/streptomycin formulation given above. The Jurkat cells were grown in 225 cm^2 flasks (Costar) at 10^6 cells/ml, containing 200 ml of medium.

B. Preparation of cell lysates.

Cells incubated with either 100 μM tamoxifen or 10 μM staurosporine were collected at various time points, one 70 - 80% confluent (about 10^7 cells) plate for each time point. The plate was placed on ice, and all subsequent steps were performed either on ice or at 4°C. The 20 ml of media in each plate containing any detached cells was saved in a 50 ml conical centrifuge tube. The adherent cells received 20 ml of PBS and were then gently lifted off the plate with a cell scraper and pooled with the detached cells. A final 10 ml was used to completely wash off the plate. The combined 50 ml was placed on ice and treated with the protease inhibitor cocktail Complete (Boehringer Mannheim, Germany) to arrest further protease activity. Cells were pelleted at 400 g for 5 min at 4°C, washed again in 15 ml of PBS treated with Complete, and re-pelleted in a 15 ml conical centrifuge tube. The cells were then re-suspended in lysis buffer containing 62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 1% mercaptoethanol, and Complete, to a volume equal to that of the cell pellet. Lysates were boiled for 5 min and then passed through a 27-gauge needle to shear the DNA, and stored at -84°C, for later Western blot analysis.

C. Preparation of cytoplasmic extracts.

The 16,000 g cytoplasmic extract used in this work is made free of nuclei, mitochondria, and any contaminating apoptotic inducing agent (such as cytochrome c or AIF which can be released from damaged mitochondria), that would lead the extract to self-prime. For adherent cells, between 2 and 40 plates were harvested. The required plates were removed from the incubator and immediately placed on ice, and all subsequent steps were performed either on ice or at 4°C. The 20 ml of media in a plate was removed and discarded, and another 10 ml of ice cold PBS (pH 7.2) was added to the plate. Note that extract of apoptotic cells was made

before appreciable cell detachment. The cells were then gently, but quickly, lifted off the plate with a cell scraper, and placed on ice in a 50 ml centrifuge tube until all cells could be harvested for centrifugation. The cells were centrifuged (4°C) at 200 g and the supernatant removed by aspiration. The resulting cell pellet was washed twice in 50 ml of ice cold PBS. The cells were then re-suspended in a 15 ml conical centrifuge tube with 10 ml of hypotonic extraction Buffer [(HEB); 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 μM cytochalasin B, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)], formulated to swell the cells in preparation for homogenization. The cells were centrifuged at 1000 g (4°C) to form a tight pellet. The supernatant was aspirated, and the volume of the cell pellet was approximated.

Next, HEB was added to a volume between 0.6 - 1 times the pellet volume. The cells were transferred to a 2 ml Dounce homogenizer (Kontes Glass Company) and allowed to swell for 20 - 30 min on ice. Cells were lysed with 20 - 100 gentle strokes of a B-type pestle. The desired extent of lysis (> 90%) was monitored under the microscope by Trypan blue staining. The volume of HEB added, the time of swelling, and the number of pestle strokes all varied according to the cell type. The cell lysate was then transferred to an Eppendorf tube and centrifuged for 30 min at 16,000 g (4°C) in an Eppendorf 5415 C microcentrifuge. The clarified supernatant was carefully removed, leaving approximately 50 μl behind, so as not to contaminate the extract. The extract was used immediately or stored in aliquots at -84°C. Extracts made this way from primed or apoptotic cells lost little, if any, of their apoptosis inducing activity.

The 3,000 g cytoplasmic extract used in this work contains mitochondria, along with pieces of plasma membrane etc., but not whole cells, or nuclei. The method here is the same as that for 16,000 g extracts, with the following distinctions. The lysis buffer is the cell free system buffer (CFS) used by Zamzami *et al.*, J. Exp. Med., 183:1533-1544 (1996) [CFS; 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, 2 mM ATP, 50 μg/ml creatine phosphokinase, 10 mM phosphocreatine, 1 mM DTT and 10 mM Hepes-NaOH, pH 7.4.], supplemented with the protease inhibitors leupeptin (1 μg/ml), pepstatin A (1 μg/ml), antipain (50 μg/ml), and chymostatin (10 μg/ml). This buffer is designed not to be hypotonic, so mitochondria do not swell. Yet, at the same time, the cells do not swell either. Of the cell lines described herein, CSM cells were the only cell line big enough to lyse in the homogenizer without swelling. After lysis of the cells, the lysate was centrifuged for 5 min at 1,000 g (4°C) to remove whole cells and most of the nuclei. Second, the supernatant was then centrifuged for 5 min at 3,000 g (4°C). These extracts were used immediately to avoid mitochondrial uncoupling when frozen. The protease inhibitors were purchased from Boehringer Mannheim, Germany.

D. Protein determination.

The Pierce Coomassie Plus protein assay with BSA standard was used to assay protein concentration in cell extracts using a Shimadzu UV-2101 PC UV-Vis Scanning Spectrophotometer. Typically, 16,000 g extracts had a protein concentration between 15 - 20 mg/ml, and 3,000 g extracts between 25 - 30 mg/ml.

E. Preparation of nuclei.

Rat liver nuclei were prepared as described (Newmeyer *et al.*, Cell, (1994)). CSM and HeLa nuclei were prepared as described (Martin *et al.*, EMBO J. 14:5191-5200 (1995b)).

F. Preparation of mitochondria.

Rat and mouse liver mitochondria were prepared as described by Hovius *et al.*, Biochem. Biophys. Acta, 1021:217-226 (1990) with some modifications. Adult Sprague-Dawley rats (~250 g) or adult BalbC mice (~50 g) were fasted for 6 h, and then killed by CO_2 inhalation (or cervical dislocation). The livers were quickly removed and submerged in ice cold mitochondria isolation buffer (MIB) [MIB; 250 mM mannitol (or sometimes 210 mM mannitol and 70 mM sucrose), 0.5 mM EGTA, 5 mM Hepes, 0.1 - 0.05% (w/v) bovine serum albumin (BSA) (pH 7.2)], supplemented with the protease inhibitors of leupeptin (1 μg/ml), pepstatin A (1 μg/ml), antipain (50 μg/ml), and chymostatin (10 μg/ml). The entire mitochondria isolation was performed on ice or at 4°C. The livers were washed in MIB to remove as much blood as possible, and then chopped into 1 - 2 mm^2 cubes with a razor blade. The small cubes were then rinsed off in a sieve (Science Ware Mini-Sieve Microsieve set; Fisher), and transferred to a 15 ml Potter Elvehjem Homogenizer (Kontes Glass Company) which was surrounded by ice.

Using a tight fitting Teflon-coated pestle mounted in a cordless drill, the tissue was homogenized by 6 - 10 up and down strokes at approximately 1750 rpm. Light microscopy (Nikon LABOPHOT-2) with Trypan blue staining was used to monitor the extent of cell lysis (usually >90%). Large cell debris and nuclei were pelleted by centrifuging twice for 5 min at 600 g in a Beckman JA-12 or JA25.50 rotor. Mitochondria were pelleted by centrifuging the supernatant for 10 min at

10,300 g in the same rotor. The crude mitochondrial pellet was further processed by gradient (isopycnic) centrifugation in Percoll. After suspending the pellet in 5 ml of MIB, the suspension was loaded on a continuous Percoll gradient. The gradient was made from the diffusion of 4 discontinuous layers (25%, 35%, 45% and 60%) of Percoll in MIB for 3 - 4 h at 25°C, which was then placed at 4oC until needed. The suspension/gradient was then centrifuged at 40,000 g for 40 min using a Beckman JA-12 or JA25.50 rotor. The gradient also contained of 250 mM mannitol, 1 mM EGTA, 25 mM Hepes, 0.1% BSA (pH 7.4), as an osmotic balancer. The mitochondria were removed from the brown band at approximately 1.10 g/ml with a Pasteur pipette. Sometimes the discontinuous gradient method of Boutry and Briquet, Eur. J. Biochem. 127:129-136 (1982) was used in place of the above continuous gradient technique. The mitochondrial pellets were washed with MIB by centrifuging for 10 min at 6300 g in a Beckman JA-12 or JA25.50 rotor. The mitochondria were then gently suspended in mitochondria storage buffer (MSB) [MSB; 400 mM mannitol, 10 mM KH_2PO_4, 5 mg/ ml BSA, 50 mM Tris-HCl, pH 7.2), and stored on ice for up to 4 h, until needed for the experimentation. Cultured cell mitochondria were prepared as described previously (Moreadith and Fiskum, Anal. Biochem. 137:360-367 (1984) incorporated herein by reference in its entirety).

G. Activation of cell-free apoptosis.

The assay of apoptotic substrate cleavage, DNA fragmentation, and caspase activity in cell-free reactions involved the formulation of the following cell-free systems:

For reactions with primed apoptotic extracts, two systems were reconstituted. First, reactions of

primed (or apoptotic) extract on nuclear substrates: 20 μl of normal, primed or apoptotic 16,000 g extract (15 - 25 mg/ml protein), 1 μl of nuclei (2 x 10^5), and 4 μl of HEB buffer or synthetic inhibitor peptides diluted in this buffer to a total volume of 25 μl. Second, reactions of primed (or apoptotic) extract on cytosolic substrates: 20 μl of normal cytoplasmic extract (15 - 25 mg/ml protein), 5 μl of primed or apoptotic cytoplasmic extract (15 - 25 mg/ml protein), and 4 μl of HEB buffer, or synthetic peptides diluted in this buffer.

For reactions activated by cytochrome c and dATP, a system was reconstituted according to the following formula: 10 μl of 16,000 g normal extract, 0.1 μl cytochrome c (1 - 10 μM final), and 0.1 μl of dATP (10 mM final), 1 - 2 μl of peptide (or other) inhibitors or HEB buffer, and 0.5 - 1 μl of nuclei (2 x 10^5) or HEB buffer.

For cell-free reactions activated by mastoparan or atractyloside, two systems were reconstituted. First, reactions involving 3,000 g extracts: 20 μl normal extract, and 2 μl of atractyloside (5 mM final concentration) or mastoparan (10 - 50 μM final concentration), or CFS buffer. Second, reactions involving 16,000 g extracts: In these reactions, mitochondria, purified by one of the methods above, were washed once after the purification and suspended in CFS to 5000 ng/ml. The ionic strength/osmolarity of the extract was then altered to account for the 50% of the extract composed of hypotonic buffer by adding to every 10 μl of extract, 0.5 μl of a 10x stock of CFS buffer. The mitochondria were then added to extract with mastoparan or atractyloside according to the following formula: 20 μl normal extract, 2 μl of mitochondria (final concentration of 500 ng/ml), and 2 μl of

atractyloside (5 mM final concentration) or mastoparan (10 - 50 μM final concentration) or CFS buffer.

For cell-free reactions activated by a mastoparan-treated mitochondrial fraction, a system was reconstituted according to the following formula: In this experiment, reactions just like that in the section below *Release of cytochrome c from mitochondria* were run, but then the supernatants were mixed with normal extract according to the following formulation: 20 μl normal extract, 5 μl of supernatant. The supernatant was concentrated down from 50 μl of supernatant to 5 μl using a 3,000 Da cut-off protein Micro-concentrator (available from Amicon).

All of the above cell-free reactions were then carried out in 500 μl o-ring sealed, screw-top microcentrifuge tubes (Continental Biological Supply), in a heat bath at 30°C or 37°C for various time periods. At the end of the incubation period, reaction tubes were flash frozen on dry ice, or in liquid nitrogen, and stored at -84°C.

N-benzyloxycaronyl-Val-Ala-Asp.fluoromethylketone (zVAD.fmk) was purchased from Enzyme Systems, Dublin, CA.. Ac-YVAD aldehyde and Ac-DEVD-adehyde were purchased from BACHEM Bioscience Inc. Bovine heart cytochrome c, horse heart cytochrome c, yeast (ISO-1) cytochrome c, and partially acetylated cytochrome c were purchased from Sigma Chemical Company. In addition, bovine heart cytochrome c and horse heart cytochrome c were purchased from Fluka. dATP was purchased from Promega and Gibco. Sodium and potassium atractyloside, mastoparan were purchased by Sigma Chemical Company. Control peptide (DLSLARLATAR- LAI) was purchased from Coast Scientific, San Diego, California.

H. Quantification of apoptosis.

For adherent cells undergoing early apoptosis morphological changes associated with apoptotic cell death were monitored using an acridine orange/ethidium bromide solution by the method described in McGahon, *et. al.*, Methods in Cell Biology, 46:153-185 (1995).

I. Protein electrophoresis and Western blots.

Electrophoresis of proteins was carried out using either 8% or 12% SDS-polyacrylamide gels. Prior to loading samples on the gel, bromophenol blue dye was added to each sample (0.002% final concentration). Equal amounts of total protein were loaded per lane, and proteins were separated at 4°C under reducing conditions at 50 V through the stacking gel, and 90 V through the separating gel.

Western blot transfer of separated proteins was carried out at 4°C using PVDF Membranes (0.2 *mm*) (Biorad), at either 120 mA overnight or 200 mA for 2 hours. Blots were then blocked for 1 h in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween) containing 5% non-fat dried milk, in polyethylene Seal-A-Meal bags. Next, the membranes were probed with an appropriate dilution (1:500 to 1:2000) of primary antibody in TBST containing 5% non-fat dried milk for 1-4 h, depending upon the antibody.

Anti-α-fodrin mouse monoclonal antibody was purchased from Chemicon International. Anti-δPKC-d rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Anti-poly (ADP-ribose) polymerase (PARP) rabbit polyclonal antibody was purchased from BIOMOL Research Laboratories, Inc., Anti-CPP32 mouse

monoclonal antibody was purchased from Transduction Laboratories, Inc. Anti-CPP32 rabbit polyclonal antibody was purchased from Upstate Biotechnology, Inc. Anti-CPP32 goat polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. Anti-lamin B mouse monoclonal mouse antibody was purchased from Oncogene Research Products (CALBIOCHEM). Anti-cytochrome c mouse monoclonal antibody was provided by Dr. Ronald Jemmerson (University of Minnesota Medical School, Minneapolis, Minnesota 55455). Human sera containing highly specific, high titer auto-antibodies to poly (ADP-ribose) polymerase (PARP), DNA topoisomerase I, U1-70 kDa, NuMa, Lamin B, Jo-1, rRNP, and PCNA were from the collection of W.M. Keck Autoimmune Disease Center Laboratory (The Scripps Research Institute, La Jolla, CA) serum bank (Casiano *et al.*, J. Exp. Med. 184:765-770 (1996) incorporated herein by reference in its entirety).

The blots were then washed for 1 h with frequent changes of TBST, followed by incubation in a peroxidase-coupled secondary antibody for 1 h in TBST containing 5% non-fat dried milk, in a Seal-A-Meal bag. The mouse, human, and rabbit peroxidase-coupled secondary antibodies were from Amersham. The blots were washed for 1 h with frequent changes of TBST. Enhanced chemiluminescence detection of the proteins was carried out using Hyperfilm ECL (Amersham), and with Pierce SuperSignal Substrate Western Blotting reagents, or Amersham ECL reagents.

J. Internucleosomal DNA fragmentation.

After incubation in the cytoplasmic extracts, for various time periods, nuclei were lysed in TE buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) containing 0.5% sodium lauryl sarkosyl and 0.5 mg/ml proteinase K. Digestion was continued for 1-3 h at 50°C, followed by

the addition of Rnase A to 1.0 mg/ml and further incubation for 1h. Running dye (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol) was then added in 1 :6 ratio of dye: sample, and the DNA preparations were electrophoresed in 1.5 - 2 % agarose gels in TAE buffer (40 mM Tris-acetate, 1mM EDTA), or TBE buffer (40 mM Tris-borate, 1 mM EDTA), at 4V per cm of gel (30 - 35 V), in a Biorad Mini-Sub Cell electrophoresis apparatus. DNA was visualized by ethidium bromide staining.

K. Measurement of caspase activation and activity.

For the caspase activation assay, the following cell-free system was reconstituted. First, a 100 μl aliquot (at least) of 16,000 g extract was clarified a second time for 20-30 min at 4°C, to ensure reproducable spectrophotoretric results of fully clarified extract. Next, 1 μl of DEVD-pNA, or YVAD-pNA substrate (100 μM final concentration) was added to 50 μl of the 16,000 g normal extract (15 - 25 mg/ml protein), and allowed to come to thermal equilibrium for 2 - 3 min at either 30°C or 37°C in a well of a Coring 96-well plate placed in a Molecular Devices MAXline Microplate Spectrophotometer (Molecular Devices, Ca, USA). Next, 1 μl of cytochrome c (1 - 10 μM final concentration) and 1 μl of dATP (1 mM final concentration) were then added, along with 1 ml of inhibitor, or buffer. Hydrolysis of the substrate was followed spectrophotometrically at 405 nm for 30-60 min at either 30°C or 37°C.

For the study of caspase activity, cell-free system was reconstituted according to the following formula: 10 μl of 16,000 g normal extract, 0.1 μl cytochrome c (1 - 10 μM final), and 0.1 μl of dATP (1 mM final), 1 - 2 μl of peptide (or other) inhibitors or HEB buffer, and 0.5 - 1 μl of nuclei (2 x 105) or HEB buffer. The reactions were run as in Example I.G.

(Activation of cell-free apoptosis). After the incubation was complete, a 1 - 8 μl aliquot of extract from a cell-free reaction was added to 100 μl of assay buffer (Assay buffer: 10% sucrose, 50 mM Hepes, 100 mM NaCl, and 0.1% CHAPS pH 7.4.) containing 100 μM DEVD-pNA N-acetyl-Asp-Glu-Val-Asp-pNA. Hydrolysis of the substrate was followed spectrophotometrically at 405 nm for 30-60 min at either 30°C or 37°C.

10 mM stocks of the substrates DEVD-pNA (sequence; N-acetyl-Asp-Glu-Val-Asp-pNA (p-nitroanilide); (Nicholson *et al.*, Nature, 376:37-43 (1995); Lazebnik *et al.*, Nature, 371:346-347 (1994)), and YVAD-pNA (sequence; N-acetyl-Tyr-Val-Ala-Asp-pNA; (Reiter, Inter. J. Peptide and Prot. Res. 43:87-96 (1994)), were made in DMSO, and were purchased from BIOMOL Research Laboratories, Inc.

Example 2
Pre-Mitochondrial Activation of Neural Apoptosis

This Example demonstrates that staurosporine and tamoxifen induce pre-mitochondria-dependent neuronal cell-free apoptosis.

A. Activation of neural apoptosis with staurosporine.

This example demonstrates that the kinase inhibitor staurosporine (Koh *et al.*, Exp. Neurol. 135:153-159 (1995); induced a relatively rapid (> 95% apoptotic cell death within 24 h, for 10 μM staurosporine), and relatively synchronous apoptosis in the CSM-25 neural cell line, as judged by morphology (see Figure 1A). Similar results were obtained with the NSC-19 cell line.

Many of the stimuli that induce apoptotic cell death in neural cell lines induce it over several days and result in mixed populations of apoptotic and viable cells. Furthermore, the dying cells are in various stages of apoptosis. For example, 100 *m*M H_2O_2, 10 min UV exposure, withdrawal of trophic factors, and serum withdrawal, all induce slow neural cell death (> 30 h for 50% cell death) (Bredesen, Cold Spring Harbor Laboratory Press, 397-421 (1994); Ann. Neurol. 38:839-851 (1995), with significant numbers of cells passing through all phases of death, from early apoptosis to secondary necrosis. Such asynchronous apoptosis makes it unfeasible to make extract from cells at, or near, the same stage of cell death. Synchronous cell extracts are required to study effectively the temporal ordering of events in apoptosis (e.g., a protease cascade), or to make a so-called primed extract, which is made from cells committed to, but not yet engaged in, apoptosis, thus representing a more upstream stage of apoptosis than extracts taken from apoptotic cells. In this study, we made both primed and apoptotic extracts. For our purposes, an extract was considered primed if it met the following criteria: 1) the cells showed little or no morphological change at the time of harvest, and 2) there was little or no cleavage of the cytosolic substrate fodrin.

In order to define the appropriate conditions to prepare staurosporine-primed extracts and to define the kinetics of apoptotic cell death in CSM-25, we carried out a time course with a substrate profile of caspase cleavage events. Table 1 lists nuclear and cytosolic substrates that were found to be cleaved in staurosporine-treated neural cells, and gives a brief description of the function of these proteins and their proteolytic fragments.

Table 1. Proteolysis of some substrates in neural staurosporine-induced apoptosis.

Substrate	Function	Proteolytic fragments
	Nuclear Substrates	
DNA topoisomerase I (Topo 1)	Modification of DNA topology Liu, L.F. (1989), Ann. Rev. Biohem. *58*, 351-375.	100 kDa->70 kDa
Lamin B	Nuclear envelope formation; anchoring chromatin to nuclear matriix, Lazebnik *et al.*(1995), Proc. Natl. Acad. Sci. USA *92*, 9042-9046.	68 kDa->45 kDa
NuMA	Involved in nuclear structure, and nuclear re-formation Compton, D.A., and Cleveland, D.W. (1994), Curr. Opin. Cell Biol.*6*, 343-6.	210 kDa->160,180 kDa
PARP	DNA repair; interaction with chromatin in the nuclear matrix Lazebnik, Y.A. *et al.* (1994), Nature *371*,346-347.	110 kDa->85 kDa
U1-70 kDa	pre-mRNA splicing Casciola-Rosen, L.A. *et al.* (1994), J. Biol. Chem. *49*, 30757-30760.	70 kDa->40 kDa
PCNA	Proliferation-associated DNA replication Bravo, R. *et al.* (1987). Nature *326*, 515-517.	Not cleaved
Ku	DNA repair Ajmani *et al.* (1995), J. Exp. Med. 181, 2049-2058.	Not cleaved
	Cytosolic Substrates	
Jo-1	his zRNA synthetase Casiano, C.A. *et al.* (1996), J. Exp. Med. *184*, 765-770.	Not cleaved
rRNP	ribosomal proteins P_0,P_1,P_2 Casiano, C.A. *et al.* (1996), J. Exp. Med. *184*,765-770.	Not cleaved
Fodrin	Cytoskeletal protein Martin, S.J. and Green, D.R. (1995c) Cell *82*, 1-20.	240 kDa->150,120 kDa

Table 1. Proteolysis of some substrates in neural staurosporine-induced apoptosis. (continued)

Substrate	Function	Proteolytic fragments
	Nuclear /Cytosolic Substrates	
PKC-d	Signal transduction; activated in apoptosis; blocked by Bcl-2 Emoto, Y. Manome, *et al.* (1995), The EMBO J. *24*, 6148-6156.	78 kDa->40 kDa

Figure 1B shows the proteolytic profile of 11 substrates in neural cell lines.

The nuclear substrate PARP was fully cleaved within the first 3 h of staurosporine-induced neural cell death, and was the first substrate fully cleaved in our kinetic profile. This is consistent with other kinetic studies of substrate cleavage events indicating that PARP is cleaved early during apoptosis (Casiano *et al.*, J. Exp. Med. 184:765-770 (1996); Tewari *et al.*, Cell 81:801-809 (1995)). After 3 h exposure to staurosporine, small amounts of cleaved PKC-δ, lamin B, U1-70, fodrin, and NuMA appeared. For the cytoplasmic substrate fodrin, cleavage was complete by 6 h, while U1-70, NuMA, PKC-δ, Topo I, and lamin B were substantially cleaved 12 h into staurosporine-stimulated apoptosis.

The cleavage events during the neural apoptosis described above were selective, in that some proteins did not undergo proteolysis; for example, the nuclear proteins PCNA and Ku, and the cytoplasmic proteins Jo-1 and rRNP, remained uncleaved after 12 h (Figure 1B), consistent with observations in non-neural cells (Casiano *et al.*, Supra).

B. Activation of neural apoptosis with tamoxifen.

This example demonstrates that tamoxifen is an extremely potent inducer of apoptosis in non-glial neural cells.

Tamoxifen is effective in treatment of estrogen receptor (ER)-positive, as well as some ER-negative, breast cancers. Although the precise mechanism of action of tamoxifen, especially in estrogen-independent cells, remains unclear, like staurosporine, it is a protein kinase C (PKC) inhibitor (Couldwell *et al.*, FEBS Lett., 345:43-46 (1994) and Neurosurgery 35:1184-1186, (1996), and such inhibition is known to induce apoptosis (Stanwell *et al.*, C. Carcinogenesis, 17:1259-1265 (1996)). The tamoxifen-mediated activation of apoptosis has been reported in the human glioblastoma cell line WITG3 (Iwasaki *et al.*, Cancer Immun. Immunother., 40:228-234 (1995)), and in some non-neural cell lines: rat osteoclasts T-289 melanoma cell line and the estrogen receptor positive MCF-7 and estrogen receptor negative MDA-231 human mammary carcinoma cell lines.

Tamoxifen citrate, 4-hydroxytamoxifen, and tamoxifen, were purchased from Sigma Chemical Company. Cells were induced to undergo apoptosis by exposure to 100 μM tamoxifen citrate (or 4-hydroxytamoxifen, or tamoxifen) for times ranging from 1 - 24 h at the incubation temperature of the cell line. The treatment of non-glial, neural CSM-25 (and NSC) cells with 100 μM tamoxifen resulted in the rapid activation of apoptosis (~100% in 3 h) (Figure 1A). More importantly, tamoxifen produced a homogeneous detachment of neural cells in about 2 h. Cells were in late apoptosis by 4 - 6 h, as judged by morphology. In contrast, with respect to staurosporine, although all of the cells rounded up within about 1 h, the cells detached at various times, over several hours, and did not exhibit the classic progressive morphological changes in synchrony. For this reason primed cytoplasmic extracts were also made from tamoxifen-treated cells, in addition to extracts made

from staurosporine-treated cells. It is worth noting that tamoxifen citrate and 4-hydroxy-tamoxifen were somewhat more effective than the free base.

C. Activation of neural cell-free apoptosis with primed extracts.

The results presented above indicate that CSM-25 cells treated with staurosporine or tamoxifen are well into apoptotic cell death by 4 - 5 h and 2 - 3 h of treatment, respectively. Therefore, we prepared primed neural cell-free extracts made from cells incubated with staurosporine or tamoxifen for 2 - 3 h and 1 h, respectively. These extracts, labeled as 16,000 g extracts, were made free of large cellular debris, nuclei, and mitochondria. They were also made to leave the nuclei and mitochondria as intact as possible to prevent contamination of the extract by DNA, and nuclear and mitochondrial proteins. Such extracts were regularly tested by DNA electrophoresis, and Western blot probing with cytochrome c antibody to detect contamination.

It has been established in non-neural cell-free systems that certain nuclei incubated in a primed extract undergo morphological changes that faithfully reproduce those associated with apoptosis (Martin *et al.*, J. Biol. Chem. 270:6425-6428 (1995); EMBO J. 14:5191-5200 (1995); and Cell, 82:1-20 (1995)). In order to show that the neural cell-free extracts generated from tamoxifen or staurosporine treated cells were potent enough to carry out this aspect of apoptosis, HeLa, CSM, and rat liver nuclei were incubated in primed or normal extract. As shown in Figure 2A, CSM-25 nuclei incubated in NSC-19 tamoxifen-primed extract for 1.5 h at 37°C underwent nuclear changes typical of apoptosis. Initially, they appeared decondensed, with the chromatin dispersed throughout the nuclear body, and nucleolar structures were apparent. Within 30 min of incubation in the primed extracts, the nuclear chromatin started moving to the nuclear margins, forming crescent-shaped patches along the nuclear envelope, which remained intact. They then

further condensed into many discrete convex shapes over the following 60 - 90 min, and were totally destroyed in 2 h. These changes in morphology were quantified in Figure 2B, by counting apoptotic nuclei versus normal nuclei incubated in both normal and primed extracts. As this data indicates, the vast majority of nuclei incubated for up to 2 h in normal extract (non-primed) did not undergo morphological change. Similar results were obtained with staurosporine-primed extracts.

Apoptosis is usually accompanied by the cleavage of DNA at internucleosomal sites (Wyllie et al., Int. Rev. Cytol. 68:251-306 (1980)). This effect has been reproduced in several non-neural cell-free systems (Newmeyer *et al.*, Cell, 79:353-364 (1994); Lazebnik *et al.*, J. Cell. Biol. 123:7-22 (1993)). As Figure 2C illustrates, rat liver nuclei incubated in tamoxifen-primed 16,000 g CSM-25 extracts also underwent this type of chromatin destruction involving the fragmentation of DNA into integer multiples of ~ 200 bp, while nuclei incubated with normal non-primed 16,000 g neural extracts remained unfragmented for several hours.

Another hallmark of apoptosis is the selective proteolytic cleavage of key substrates (Casiano *et al.*, Supra), as discussed and illustrated above. As shown in Figure 2D, the incubation of HeLa nuclei in tamoxifen-primed 16,000 g NSC-34 extract resulted in the cleavage of PARP and PKC-δ, while the incubation of normal non-primed extract in primed extract resulted in the cleavage of fodrin. No such cleavage events were observed when normal extract was substituted for primed extract (e.g., normal extract incubated in normal extract). Comparable results were obtained with staurosporine-primed extracts. Consistent with reports on non-neural systems (Lazebnik *et al.*, Nature 371:346-347 (1994), the cleavage of PARP was blocked by Ac-DEVD-CHO (Nicholson *et al.*, Nature 376:37-43 (1995); a tetrapeptide inhibitor specific (at 1 μM) for the CPP32-like caspase family proteases, but not by Ac-YVAD-CHO (Thornberry *et al.*, Biochemistry *33*:3934-3940 (1994), a tetrapeptide inhibitor specific (at 1 μM)

for the ICE-like caspase family members. CPP32 is also known as Yama or apopain (Quan *et al.*, B. Proc. Natl. Acad. Sci. USA 93:1972-1976, (1996) Tewari *et al.*,Cell 81:801-809 (1995b).

The activation of caspases is known to be essential for apoptotic execution. The kinetics of caspase activation was measured spectrophotometrically by assaying the hydrolysis of a substrate that can only be cleaved by either a CPP32-like caspase family member (DEVD-pNA substrate) (Nicholson *et al.*, Supra), or an ICE-like caspase family member (YVAD-pNA substrate) (Thornberry *et al.*, Supra). As Figure 2E illustrates for a tamoxifen-primed 16,000 g NSC-19 extract incubated at 37°C for 2 h, only the DEVD-pNA substrate is hydrolyzed, implying that CPP32-like caspases are responsible for these selective cleavage events. Note that there is no lag phase seen in Figures 5C and 6D because this extract was incubated for 2 h before the assay, so that it was already active.

Example 3
Mitochondrial-dependent Activation of Neuronal Cell-Free Apoptosis

This Example demonstrates that mastoparan and atractyloside induce a mitochondria-dependent neuronal cell-free apoptosis, as measured by fodrin cleavage, CPP32 processing to active forms, and the CPP32-like caspase hydrolysis of the DEVD-pNA substrate.

A. Atractyloside induction of mitochondria-dependent apoptosis.

Atractyloside is an inhibitor of the mitochondrial adenine nucleotide translocator (ANT), and an inducer of the mitochondrial inner membrane permeability transition (MPT) (Zamzami *et al.*, Supra; de Macedo *et al.*, Eur. J. Biochem, 215:595-600 (1993). As shown in Figure 3, incubation of a 3,000 g CSM-25 non-primed extract for 1.5 h at 37°C in the presence of 5 mM

atractyloside resulted in the cleavage of fodrin, an event that has been shown previously to be tightly coupled to apoptosis (Martin *et al.*, Supra). Beyond this, atractyloside also induced the cleavage of fodrin in a system composed of rat liver mitochondria and a 16,000 g CSM-25 non-primed extract. However, atractyloside incubated in a 16,000 g non-primed extract alone did not lead to such cleavage, demonstrating that the cleavage of fodrin was mitochondria-dependent. Furthermore incubation of the 3,000 g CSM-25 extract alone (or the 16,000 g CSM-25 extract) did not lead to the cleavage of fodrin.

B. Mastoparan induction of mitochondria-dependent apoptosis.

The wasp venom peptide toxin mastoparan kills cultured cerebellar granular neurons by apoptosis (Yan *et al.*, J. Neurochem. 65:2425-2431 (1995). The results in Figure 4 demonstrate that mastoparan also induces cell death in cultured R2 rat cerebellar neuron precursors. This death was determined to be apoptotic within 6 h at a mastoparan concentration of 50 μM, and necrotic at a concentration of 100 μM or greater, as measured by morphology. Like atractyloside, mastoparan induces the MPT (Pfeiffer *et al.*, J. Biol. Chem. 270:4923-4932 (1995). Furthermore, mastoparan interacts with the mitochondria outer membrane to release mitochondrial proteins even before the MPT (Nicolay *et al.*, J. Bioenergetics Biomembranes 26:327-334 (1994).

Mastoparan was assayed for a mitochondria-dependent activation of protease activity unique to apoptosis. A 3,000 g CSM-25 non-primed extract was incubated with 50 μM mastoparan for 1.5 h at 37°C. The results showed that in addition to the cleavage of the cytoskeletal protein fodrin, the caspase family member CPP32 was processed to the active forms found in apoptosis (Figure 5A). Incubation of the 3,000 g CSM-25 non-primed extract alone did not lead to the cleavage of fodrin or the processing of CPP32.

Since 3,000 g non-primed extracts not only contain mitochondria, but also, for example, pieces of plasma membrane, and in light of the fact that mastoparan has been shown to interact with G proteins, it was determined whether mastoparan had indeed acted through the mitochondria. As shown in Figure 5A, mastoparan induced the cleavage of fodrin and processing of CPP32 to active forms in a neural cell-free system composed of mouse liver mitochondria in a 16,000 g NT2 normal non-primed extract, but did not prime such an extract without mitochondria (See Figure 7). A control non-helix forming peptide (DLSLARLATARLAI) did not activate these systems. In addition, the addition of CSM nuclei to these reactions resulted in nuclear morphology characteristic of apoptosis. Furthermore, the result was the same for rat neuronal mitochondria in a 16,000 g non-primed extract from primary cerebellar neurons, demonstrating the extension of the invention neural cell-free system to primary neurons (Figure 5A).

In contrast, when cell-free system buffer (CFS) was used instead of extract, there was no detectable fodrin cleavage or CPP32 processing due to the fact that there was no detectable fodrin or CPP32 in the mitochondrial preparations (by Western blot). Thus, the cytosol appears to be required for these reactions. As discussed below in Example 5, tamoxifen did not activate this system.

C. Mastoparan induces the release of cytochrome c from mitochondria.

Liu *et. al.*, Cell 86:147-157 (1996), have described a cell-free system that differs from the previously reported systems in that it uses non-primed extracts from normally growing cells that have not been induced to undergo apoptosis. Apoptosis is initiated by the addition of dATP and cytochrome c, as opposed to apocytochrome c, to extracts generated from healthy cells. In this example, whether or not mastoparan releases cytochrome c from mitochondria was determined.

As illustrated in Figure 5B, it was discovered that 50 μM mastoparan incubated for 1 h at 37°C with mouse liver mitochondria led to the release of cytochrome c (as measured by Western blot), whereas control reactions did not release cytochrome c. Furthermore, as shown in Figure 5C, it was found that the supernatant from the mastoparan treated mitochondria activated a 16,000 g normal non-primed extract made from NT2 cells, as measured by the processing of DEVD-pNA substrate. The supernatant from control reactions did not activate the 16,000 g normal non-primed extracts.

Example 4
Post-Mitochondrial Activation of Neuronal Cell-Free Apoptosis

A. Activation of neuronal cell-free apoptosis with cytochrome c and dATP.

As discussed in Example 3C Liu *et. al.* Supra, have described a cell-free system that activates extracts from normally growing cells by the addition of cytochrome c and dATP. This system, when applied to human embryonic kidney 293 cells and human monoblastic U937 cells, reproduces nuclear and DNA fragmentation, PARP cleavage, and the processing of CPP32 to active forms found in apoptotic cell death.

Ten μM horse heart cytochrome c and 1 mM dATP were added to 16,000 g neuronal/neural extracts, and it was found that this system reproduced nuclear and DNA fragmentation (Figures 6A, B), the proteolysis of fodrin and the processing of CPP32 (Figure 6C), and the activation/activity of CPP32-like caspase hydrolysis of DEVD-pNA (Figure 6D). In addition to rat primary cerebellar granule cell neurons, the system works equally well with neural cell lines such as CSM, NSC, and NT2, and non-neural cell lines such as Jurkat. In addition, as discussed in Example 5, it was found that tamoxifen and mastoparan did not activate this system.

B. Variability of cytochrome c-mediated activation of cell-free apoptosis.

This example demonstrates that some forms of cytochrome c do not activate cell-free apoptosis.

Although the neuronal cell-free system described in the previous Example 4A activates apoptosis with horse and bovine cytochrome c, it does not activate with yeast cytochrome c (ISO-1), nor with partially acetylated horse heart cytochrome c (Figure 6C). The cytochrome c acetylation process preferentially acetylates surface lysines. In common with this theme of altered lysines, yeast cytochrome c differs from mammalian cytochrome c, not only in the number and distribution of lysines (Figure 7), but also in that lysine 72 is naturally tri-methylated in yeast cytochrome c (Clements *et. al.*, Gene 83:1-14 (1989). Thus, it appears that lysines are important for the function of cytochrome c in activating cell-free apoptosis, and that mutations of these lysines might affect the mechanism by which cytochrome c initiates apoptosis.

C. Affect of Bcl-2 on cytochrome c/dATP-mediated activation of cell-free apoptosis.

This example demonstrates that cytochrome c/dATP activation of cell-free apoptosis is not inhibited by Bcl-2.

Expression of the proto-oncogene *bcl-2* inhibits both necrotic and apoptotic cell death in several cell types, including neural cells, and in response to a wide variety of inducers, including serum and growth factor withdrawal, calcium ionophores, glucose withdrawal, membrane peroxidation, glucocorticoids and chemotherapeutic agents, baculovirus infection, free radical inducing agents, and protein kinase inhibitors such as staurosporine. With respect to chronic neurodegenerative conditions such as Alzheimer's disease

and ALS, it has been shown that the expression of *bcl-2* inhibits neuronal death induced by both glutamate (Zhong *et al.*, Mol. Brain Res. *19:*353-355 (1993a) and β-amyloid peptide (Zhong *et al.*, Proc. Natl. Acad. Sci. USA *90:*4533-4537 (1993b). Furthermore, with regard to acute neurological events such as stroke, the expression of *bcl-2* protects neurons during acute *in vivo* cerebral ischemia (Martinou *et al.*, Neuron 13:1017-1030 (1994). The gene *bcl-2* encodes a 26-kDa membrane-associated protein Bcl-2 that has been ultrastructurally located to the mitochondria, the nuclear membrane, and the endoplasmic reticulum.

Susin *et al.* Supra, have shown that mitochondria from *bcl-2* overexpressing cells do not release AIF when incubated with atractyloside. As Figure 6C illustrates, both a 16,000 g non-primed extract made from Bcl-2 overexpressing CSM-25 cells, and a 3,000 g non-primed extract from such cells (which therefore contains mitochondria) are activated by cytochrome c/dATP. These results demonstrate that Bcl-2 can prevent the activation of apoptosis at the mitochondrial level but not at the post-mitochondrial level. This is consistent with the fact that cytochrome c is only found in the mitochondrial intermembrane space (under normal conditions) (Brayer and Murphy, University Science Books, Chapter 3, pp 107. (1996), and that Bcl-2 is found at the contact points between the outer and inner mitochondrial membranes (Monaghan *et al.*, J. Histochem. Cytochem. 40:1819-1825 (1992); de Jong *et al.*, Cancer Research, 54:256-60 (1994).

Therefore, although Bcl-2 can prevent neural cells from undergoing apoptosis induced by such agents as tamoxifen and staurosporine (pre-mitochondrial phase), and can prevent the cell-free activation of apoptosis by such agents as atractyloside (mitochondrial phase), it cannot prevent the cell-free initiation of neural apoptosis by cytochrome c/dATP (post-mitochondrial phase).

D. Affect of Bcl-2 on cytochrom c/dATP-mediated activation of cell-free apoptosis.

This example demonstrates that extract pre-incubation inhibits cytochrome c/dATP-activated cell-free apoptosis.

A 30 min pre-incubation of 16,000 g normal non-primed extract at 37°C renders the extract incapable of activation by cytochrome c/dATP, indicating that a temperature sensitive protease or activator is involved in this apoptotic process (Figure 6C). Indeed, reactions appeared to run better at 30°C than at 37°C, as measured by the extent of substrate cleavage, and pNA hydrolysis. Furthermore, the peptide inhibitor Z-Val-Ala-Asp-fluoro-methylketone (zVAD-fmk) of caspase proteases prevents the activation (Figure 6C). Finally, cytochrome c alone was usually enough to activate a 16,000 g non-primed extract, without the need for added dATP, although the resulting activity was lower.

Example 5

CPP32 processing in mitochondria-dependent activation of cell-free apoptosis.

The proteolytic conversion of pro-CPP32 to active forms is a hallmark of apoptosis (Casciola-Rosen *et al.*, J. Exp. Med. 183:1957-1964 (1996). The processing of CPP32 in the neural cell-free system induced by tamoxifen, mastoparan, and cytochrome c/dATP is illustrated in Figure 7.

For the data shown at the pre-mitochondrial level, while neural cells (either CSM, NSC or NT2) were incubated at 37°C with 100 μM tamoxifen, or 50 μM mastoparan, or 10 μM cytochrome c and 1 mM dATP for 2, 6, and 8 hours, respectively. While the extracts made from cells incubated with tamoxifen and mastoparan contained processed CPP32, extract made from cells incubated with cytochrome c/dATP did not.

For the data shown at the mitochondrial level, a cell-free system composed of 16,000 g neural non-primed extract and added rat liver mitochondria was incubated at 37°C with 100 μM tamoxifen, or 50 μM mastoparan, or 10 μM cytochrome c and 1 mM dATP for 2, 1, and 1 hours, respectively. Although mastoparan and cytochrome c/dATP activated the cell-free system, tamoxifen did not.

For the data shown at the post-mitochondrial level, a cell-free system composed of 16,000 g neural extract was incubated at 37°C with 100 μM tamoxifen, or 50 μM mastoparan, or 10 μM cytochrome c and 1 mM dATP, for 2, 2, and 1 hours, respectively. Although cytochrome c/dATP activated the cell-free system, tamoxifen and mastoparan did not.

The results show that tamoxifen induces apoptosis at the pre-mitochondrial level (cells), but does not induce apoptosis at the mitochondrial level (mitochondria and extract), or the post-mito-chondrial level (extract). Mastoparan induces apoptosis at the pre-mitochondrial level (cells) and at the mitochondrial level (mitochondria and extract), but does not induce apoptosis at the post-mitochondrial level (extract). And finally, cytochrome c/dATP induces apoptosis at the mitochondrial level (mitochondria and extract) and at the post-mitochondrial level (extract), but does not induce apoptosis at the pre-mitochondrial level (cells).

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

That which is claimed is:

1. A cell-free method for identifying a compound that inhibits mitochondria-dependent apoptosis, said method comprising:

a) providing a non-activated cell extract, wherein said cell extract further comprises mitochondria, and a test-compound;

b) contacting said cell extract with a mitochondria-dependent apoptosis-inducing agent; and

c) identifying a compound that inhibits apoptosis.

2. The method of claim 1, wherein said cell extract is neuronal.

3. The method of claim 2, wherein said mitochondria-dependent apoptosis-inducing agent is a mitochondria-membrane disrupting agent.

4. The method of claim 3, wherein said mitochondria-membrane disrupting agent is selected from mastoparan or atractyloside.

5. The method of claim 2, wherein said inhibition of apoptosis is determined by detecting a decrease in caspase activation.

6. The method of claim 5, wherein said caspase activation is mediated by CPP32.

7. The method of claim 2, wherein said inhibition of apoptosis is determined by detecting a decrease in caspase processing.

8. The method of claim 2, wherein said inhibition of apoptosis is determined by detecting a decrease in cytosolic substrate cleavage.

9. The method of claim 8, wherein said substrate is selected from fodrin or PKC-δ.

10. The method of claim 2, wherein said cell extract is substantially free of non-mitochondria membrane organelles.

11. The method of claim 2, wherein said mitochondria is obtained from a different cell-type than said cell extract.

12. The method of claim 2, wherein said mitochondria is obtained from the same cell-type as said cell extract.

13. The method of claim 2, wherein said cell extract further comprises at least one nuclei.

14. The method of claim 13, wherein said inhibition of apoptosis is determined by detecting decreased chromatin condensation.

15. The method of claim 13, wherein said inhibition of apoptosis is determined by detecting decreased fragmentation of the nuclei.

16. A cell-free method for identifying a compound that activates mitochondria-dependent apoptosis, said method comprising:

a) providing a non-activated cell extract containing mitochondria;

b) contacting said cell extract with a test-compound; and

c) identifying a compound that activates mitochondria-dependent neuronal apoptosis.

17. The method of claim 16, wherein said cell extract is neuronal.

18. The method of claim 17, wherein said activation of apoptosis is determined by detecting an increase in caspase activation.

19. The method of claim 18, wherein said caspase activation is mediated by CPP32.

20. The method of claim 17, wherein said activation of apoptosis is determined by detecting an increase in caspase processing.

21. The method of claim 17, wherein said activation of apoptosis is determined by detecting an increase in cytosolic substrate cleavage.

22. The method of claim 21, wherein said substrate is selected from fodrin or PKC-δ.

23. The method of claim 17, wherein said cell extract is substantially free of non-mitochondria membrane organelles.

24. The method of claim 17, wherein said mitochondria is obtained from a different cell-type than said cell extract.

25. The method of claim 17, wherein said mitochondria is obtained from the same cell-type as said cell extract.

26. The method of claim 17, wherein said cell extract further comprises at least one nuclei.

27. The method of claim 26, wherein said activation of apoptosis is determined by detecting increased chromatin condensation.

28. The method of claim 26, wherein said activation of apoptosis is determined by detecting increased fragmentation of the nuclei.

29. A cell-free method for identifying a compound that modulates neuronal apoptosis, said method comprising:

a) providing a neuronal cell extract further comprising mitochondria,

b) contacting said cell extract with a test-compound, and

c) identifying a compound that modulates apoptosis.

30. The method of claim 29, wherein said compound inhibits apoptosis, and wherein said cell extract further comprises a mitochondria-dependent apoptosis inducing agent.

31. The method of claim 29, wherein said compound activates apoptosis.

32. A cell-free method for identifying a compound that inhibits mitochondria-dependent apoptosis, said method comprising:

a) providing a non-activated cell extract in the presence and absence of a test-compound, wherein said cell extract further comprises mitochondria, and a mitochondria-dependent apoptosis-inducing agent; and

b) identifying a compound that inhibits apoptosis.

33. The method of claim 32, wherein said cell extract is neuronal.

34. The method of claim 33, wherein said mitochondria-dependent apoptosis-inducing agent is a mitochondria-membrane disrupting agent.

35. The method of claim 34, wherein said mitochondria-membrane disrupting agent is selected form mastoparan or atractyloside.

36. The method of claim 33, wherein said inhibition of apoptosis is determined by detecting a decrease in caspase activation in the presence of said test-compound relative to the level of caspase activation in the absence of said test-compound.

37. The method of claim 33, wherein said inhibition of apoptosis is determined by detecting a decrease in caspase processing in the presence of said test-compound relative to the level of caspase processing in the absence of said test-compound.

38. The method of claim 33, wherein said inhibition of apoptosis is determined by detecting a decrease in cytosolic substrate cleavage in the presence of said test-compound relative to the level of cytosolic substrate cleavage in the absence of said test-compound.

39. A method for identifying a compound that specifically modulates mitochondria-dependent apoptosis, said method comprising:

(1) determining whether a test-compound modulates mitochondria-dependent apoptosis;

(2) determining whether said test-compound modulates pre-mitochondria-dependent apoptosis;

(3) determining whether said test-compound modulates post-mitochondria-dependent apoptosis; and

(4) identifying a compound that modulates mitochondria-dependent apoptosis.

40. The method of claim 39, wherein said compound does not modulate pre-mitochondria-dependent or post-mitochondria-dependent apoptosis.

41. The method of claim 40, wherein said cell extract is neuronal.

42. A method for identifying a compound that modulates a specific mitochondria-dependent-stage of apoptosis, said method comprising:

(1) determining whether a test-compound modulates mitochondria-dependent apoptosis;

(2) determining whether said test-compound modulates pre-mitochondria-dependent apoptosis;

(3) determining whether said test-compound modulates post-mitochondria-dependent apoptosis; and

(4) identifying a compound that modulates a specific mitochondria-dependent-stage of apoptosis.

43. The method of claim 42, wherein step (4) is identifying a compound that modulates mitochondria-dependent apoptosis, and wherein said compound does not modulate pre-mitochondria-dependent or post-mitochondria-dependent apoptosis.

44. The method of claim 42, wherein step (4) is identifying a compound that modulates pre-mitochondria-dependent apoptosis, and wherein said compound does not modulate mitochondria-dependent or post-mitochondria-dependent apoptosis.

45. The method of claim 42, wherein step (4) is identifying a compound that modulates post-mitochondria-dependent apoptosis, and wherein said compound does not modulate pre-mitochondria-dependent or mitochondria-dependent apoptosis.

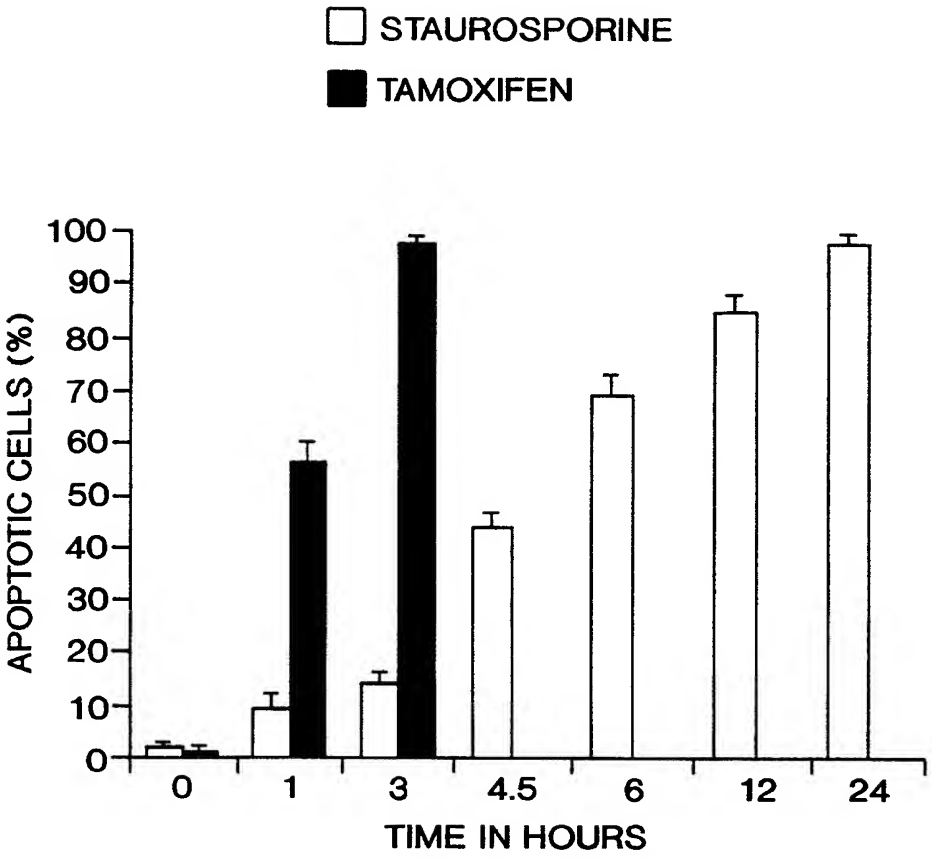

STAUROSPORINE
TAMOXIFEN
APOPTOTIC CELLS (%)
100
90
80
70
60
50
40
30
20
10
0
0
1
3
4.5
6
12
24
TIME IN HOURS

FIG. 1A

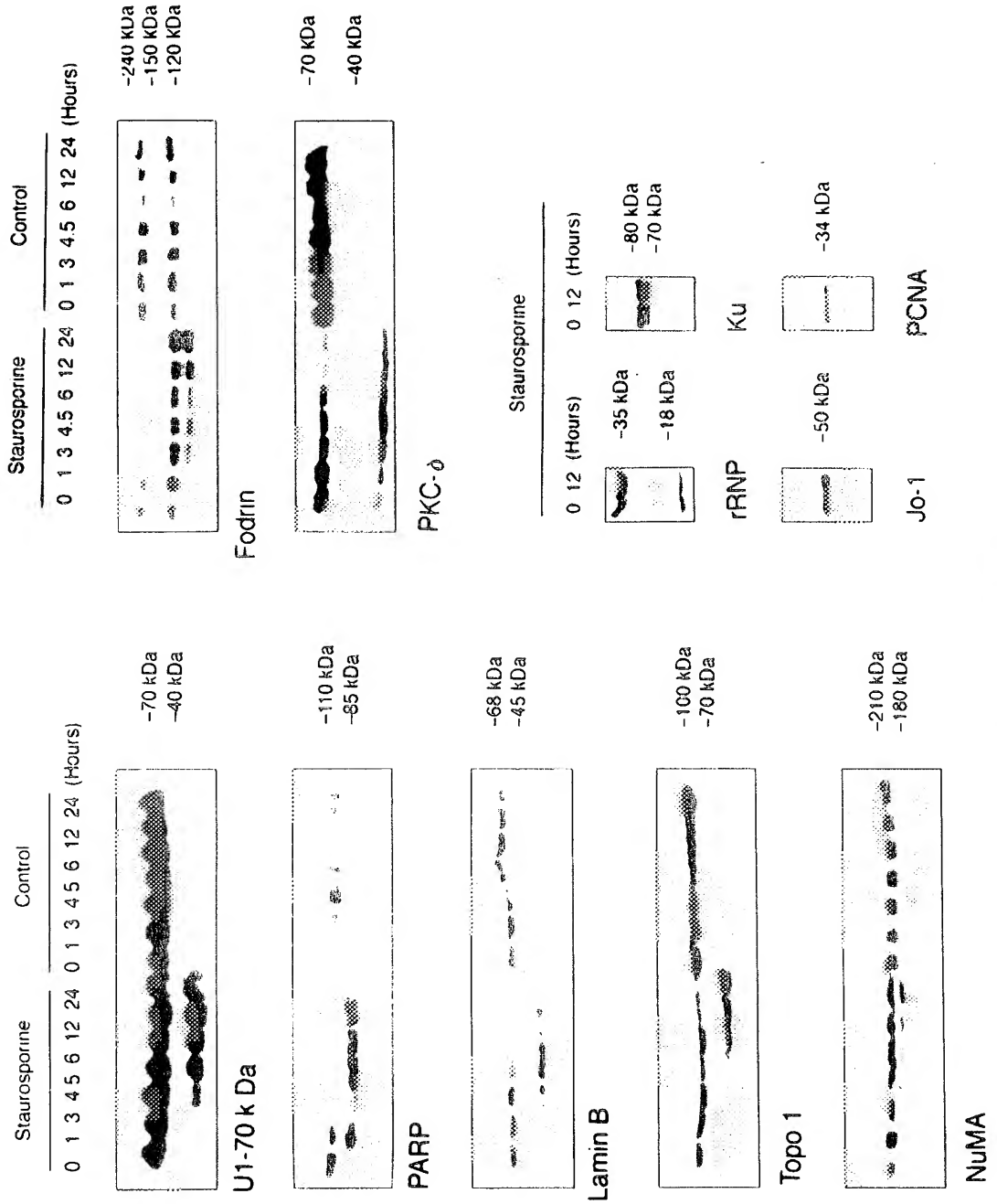

Staurosporine
Control
0 1 3 4.5 6 12 24 0 1 3 4.5 6 12 24 (Hours)
-240 kDa
-150 kDa
-120 kDa
Fodrin
-70 kDa
-40 kDa
PKC-δ
Staurosporine
0 12 (Hours) 0 12 (Hours)
-35 kDa
-18 kDa
-80 kDa
-70 kDa
rRNP
Ku
-50 kDa
-34 kDa
Jo-1
PCNA
-70 kDa
-40 kDa
U1-70 k Da
-110 kDa
-85 kDa
PARP
-68 kDa
-45 kDa
Lamin B
-100 kDa
-70 kDa
Topo 1
-210 kDa
-180 kDa
NuMA

FIG. 1B

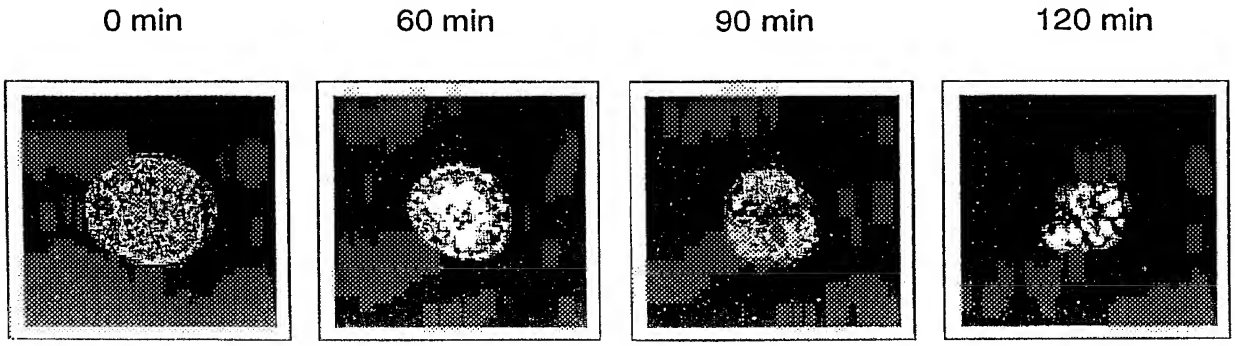

0 min
60 min
90 min
120 min

FIG. 2A

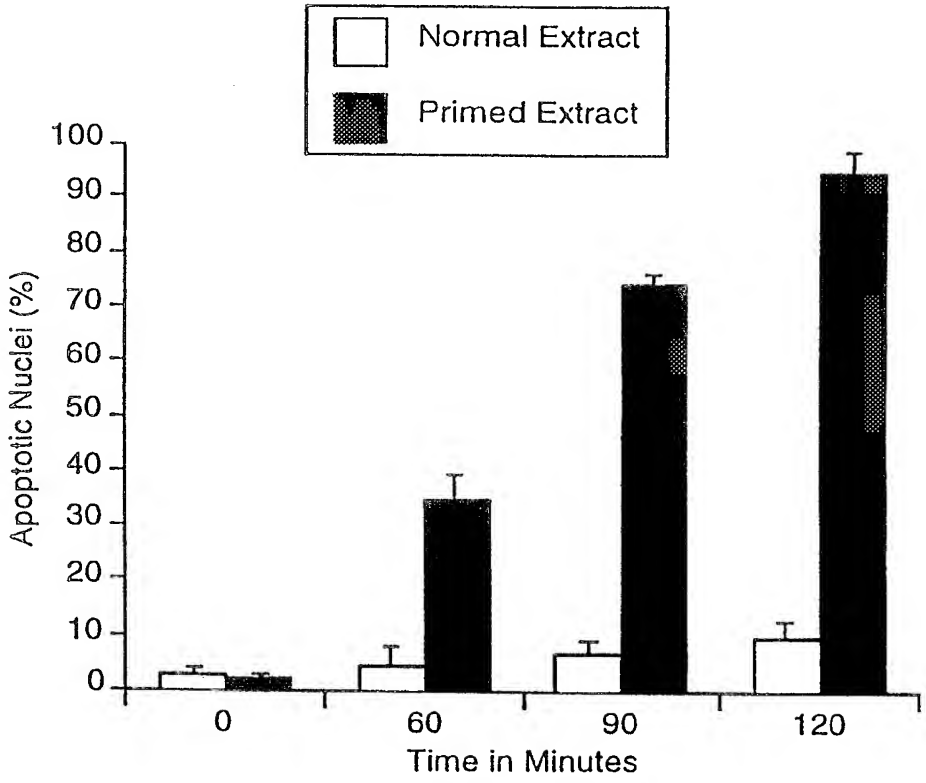

Normal Extract
Primed Extract
Apoptotic Nuclei (%)
100
90
80
70
60
50
40
30
20
10
0
0
60
90
120
Time in Minutes

FIG. 2B

Nuclei:	–	+	+	+
Normal Extract:	–	–	+	–
Primed Extract:	+	–	–	+

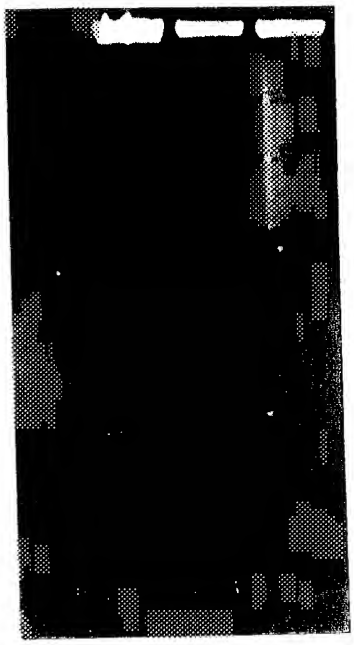


FIG. 2C

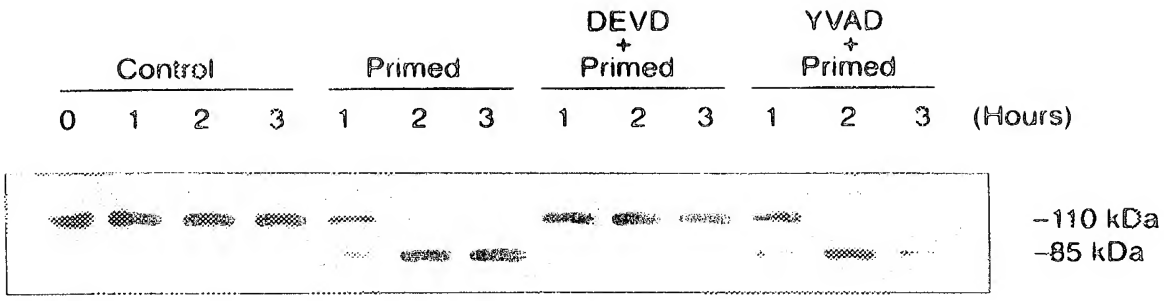


FIG. 2D

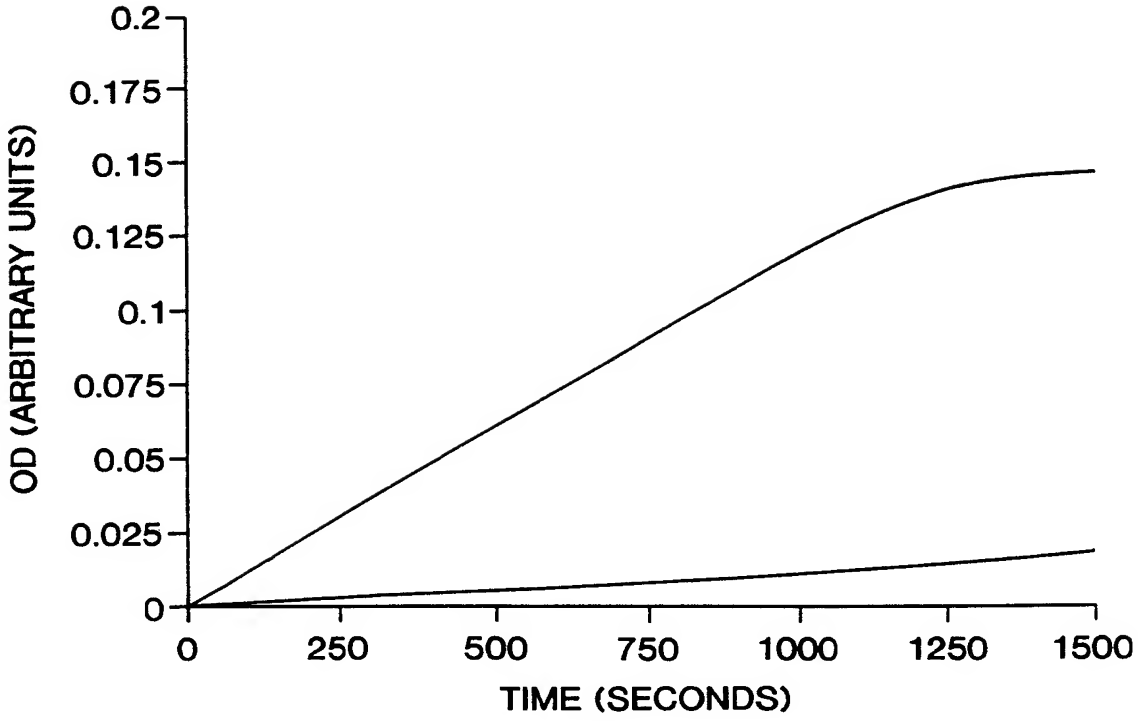
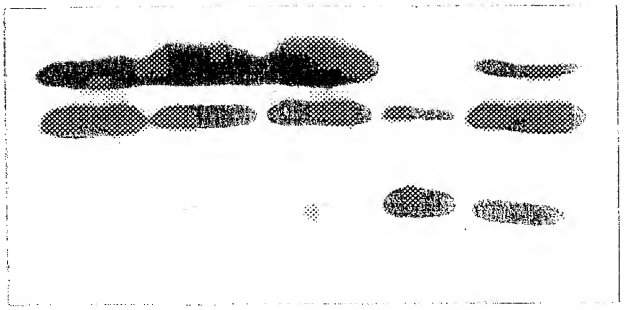

0.2
0.175
0.15
0.125
0.1
0.075
0.05
0.025
0
OD (ARBITRARY UNITS)
0
250
500
750
1000
1250
1500
TIME (SECONDS)

FIG. 2E

3,000 g Extract	+	–	–	+	–
16,000 g Extract	–	+	+	–	+
Atractyloside	–	–	+	+	+
Added Mitochondria	–	–	–	–	+



Fodrin

FIG. 3

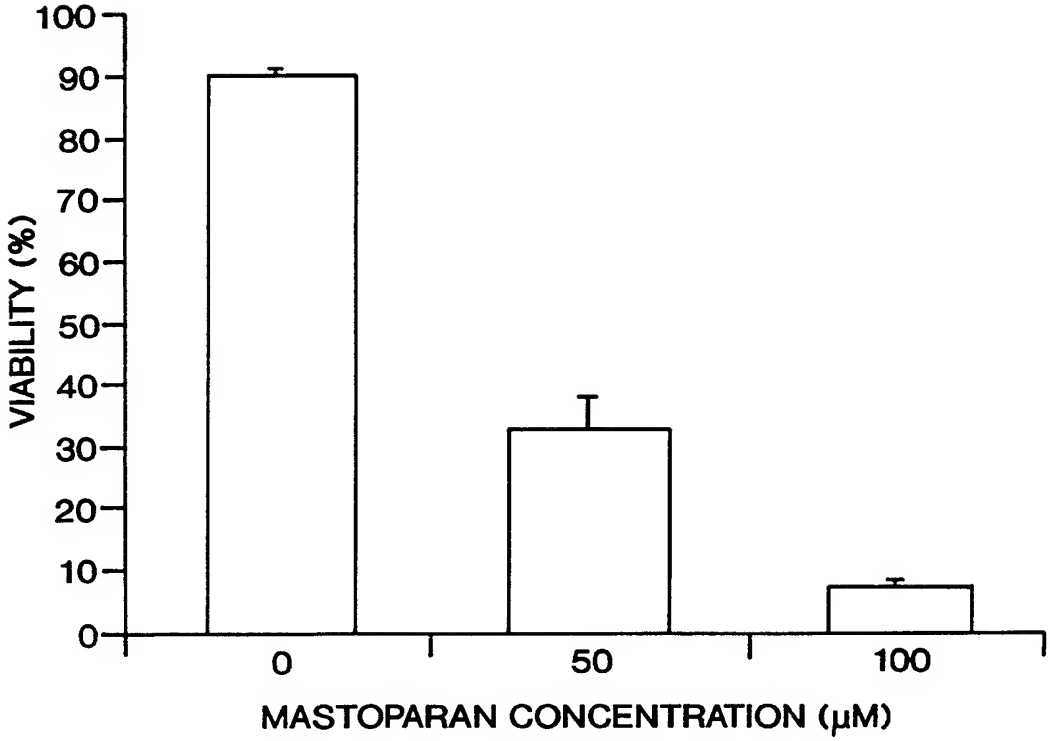

100
90
80
70
60
50
40
30
20
10
0
VIABILITY (%)
0
50
100
MASTOPARAN CONCENTRATION (μM)

FIG. 4

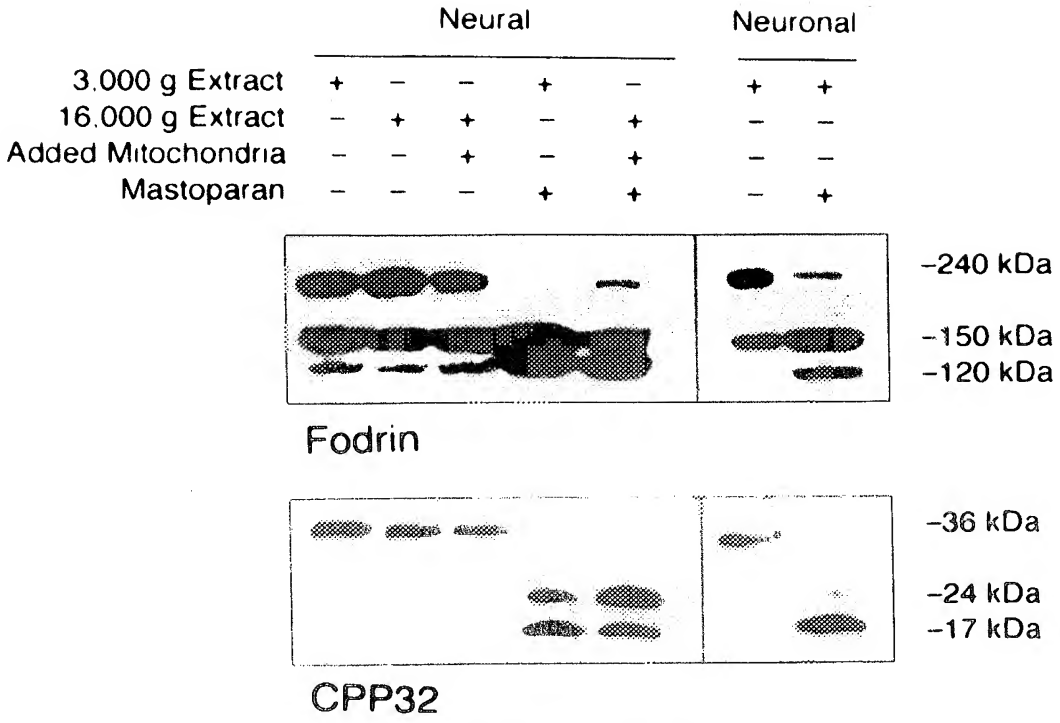

Neural
Neuronal
3,000 g Extract + − − + − + +
16,000 g Extract − + + − + − −
Added Mitochondria − − + − + − −
Mastoparan − − − + + − +
−240 kDa
−150 kDa
−120 kDa
Fodrin
−36 kDa
−24 kDa
−17 kDa
CPP32

FIG. 5A

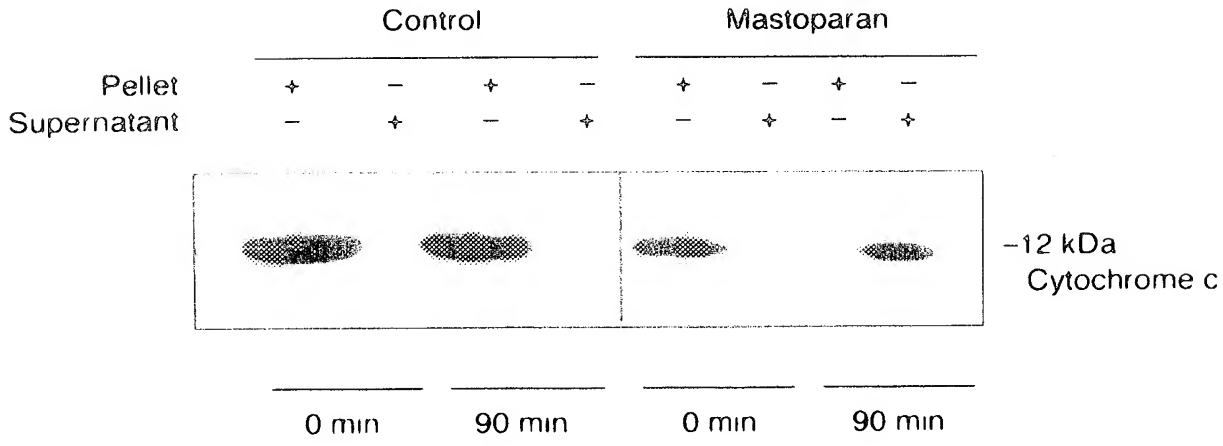

Control
Mastoparan
Pellet + − + − + − + −
Supernatant − + − + − + − +
−12 kDa
Cytochrome c
0 min
90 min
0 min
90 min

FIG. 5B

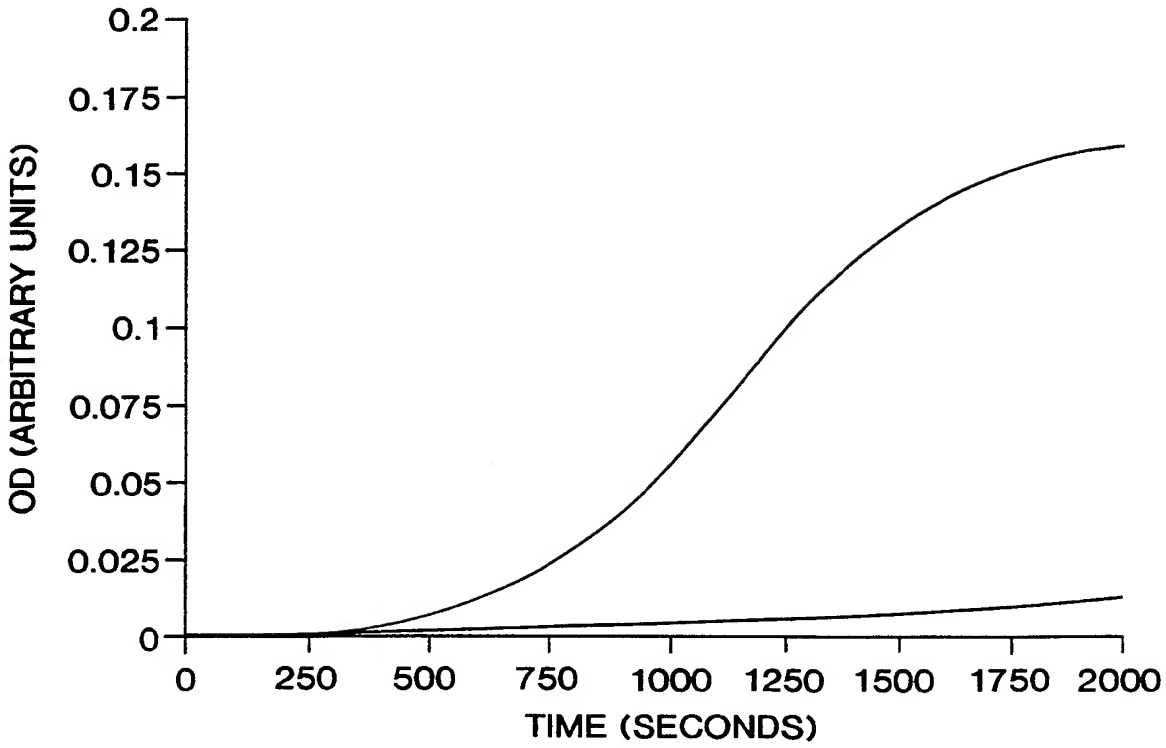
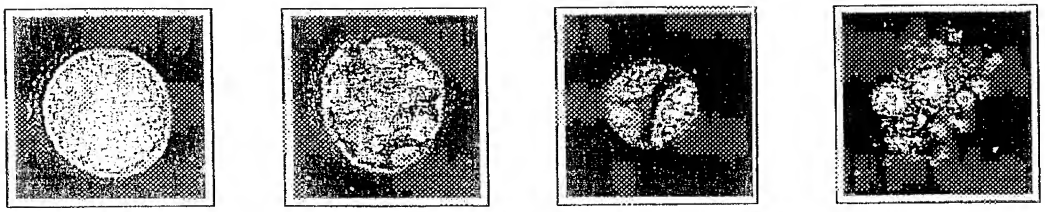

0.2
0.175
0.15
0.125
0.1
0.075
0.05
0.025
0
OD (ARBITRARY UNITS)
0
250
500
750
1000
1250
1500
1750
2000
TIME (SECONDS)

FIG. 5C



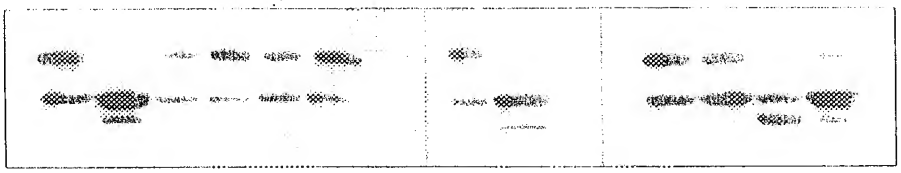
0 min 60 min 90 min 120 min

FIG. 6A

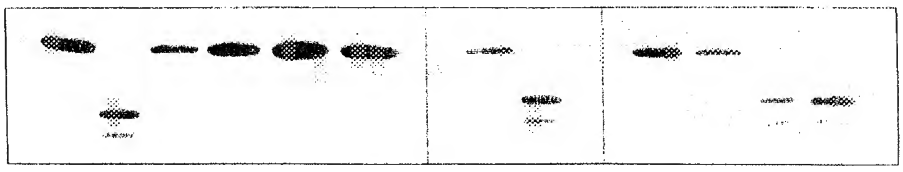
Nuclei:	–	+	+	+
Extract:	+	–	+	+
Cytochrome C/dATP:	–	–	–	+

FIG. 6B

	Neural						Neuronal		Bcl-2			
3 000 g Extract	–	–	–	–	–	–	–	–	+	–	+	–
16 000 g Extract	+	+	+	+	+	+	+	+	–	+	–	+
Cytochrome c (Horse)	–	+	–	–	+	+	–	+	–	–	+	+
Cytochrome c (Yeast)	–	–	+	–	–	–	–	–	–	–	–	–
Cytochrome c (Acetylated)	–	–	–	+	–	–	–	–	–	–	–	–
Pre-incubated	–	–	–	–	+	–	–	–	–	–	–	–
ZVAD	–	–	–	–	–	+	–	–	–	–	–	–
Added Mitochondria	–	–	–	–	–	–	–	–	–	–	–	+



Fodrin



CPP32

FIG. 6C

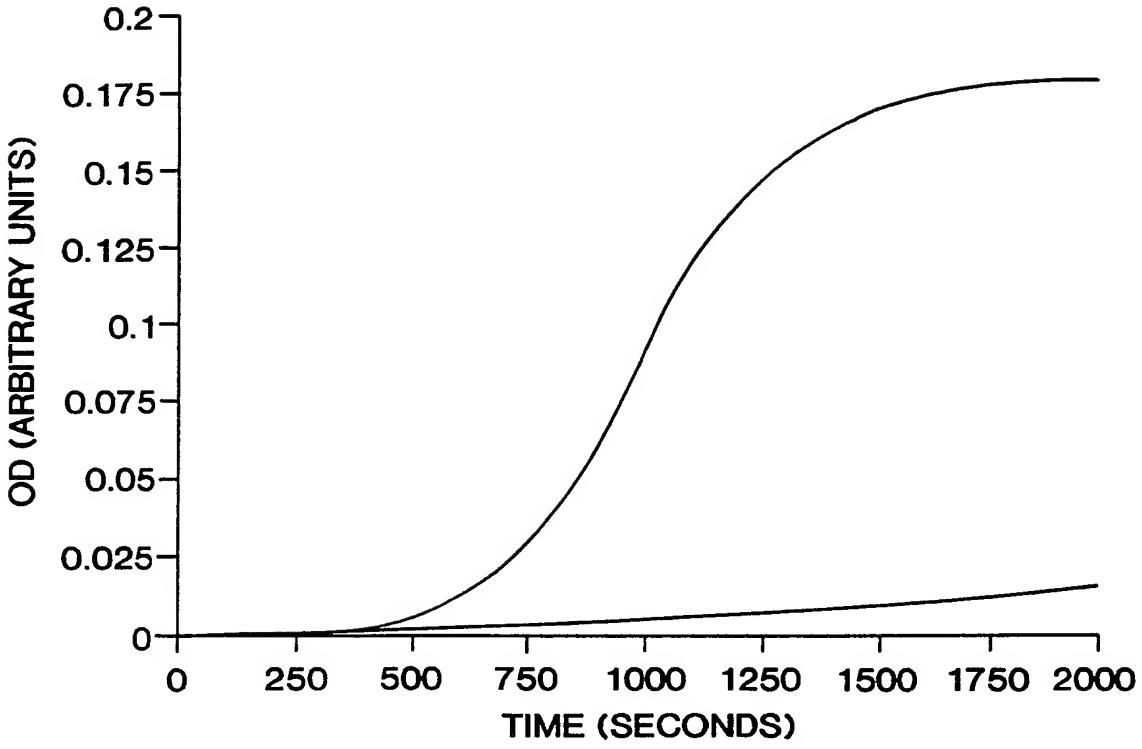

0.2
0.175
0.15
0.125
0.1
0.075
0.05
0.025
0
OD (ARBITRARY UNITS)
0
250
500
750
1000
1250
1500
1750
2000
TIME (SECONDS)

FIG. 6D

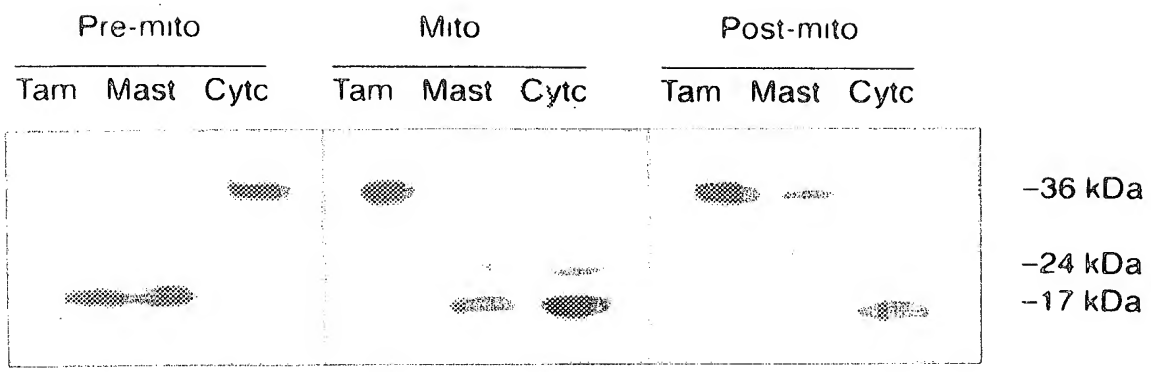

Pre-mito
Mito
Post-mito
Tam Mast Cytc
Tam Mast Cytc
Tam Mast Cytc
-36 kDa
-24 kDa
-17 kDa
CPP32

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01957

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12Q 1/00; C12N 5/06
US CL :435/4, 375
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4, 375

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	ELLERBY et al. Establishment of a Cell-Free System of Neuronal Apoptosis: Comparison of Premitochondrial, Mitochondrial, and Postmitochondrial Phases. The Journal of Neuroscience. 15 August 1997, Vol. 17, No. 16, pages 6165-6178, especially page 6165.	1-45
Y	KLUCK et al. Cytochrome c activation of CPP32-like proteolysis plays a critical role in a Xenopus cell-free apoptosis system. The EMBO Journal, 01 August 1997, Vol. 16, No. 15, pages 4639-4649, especially page 4639.	1-45

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 10 MAY 1998	Date of mailing of the international search report 06 JUL 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHN S. BRUSCA Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01957

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	ZAMZAMI et al. Mitochondrial control of Nuclear Apoptosis. J. Exp. Med. April 1996, Vol. 183, pages 1533-1544, especially page 1533.	1, 3, 8, 10, 12-16, 21, 23, 25-28 ---------- 2, 4-7, 9, 11, 17-20, 22, 24, 29-45
Y	BROWN et al. Human Papillomavirus (HPV) 16 E6 Sensitizes Cells to Atractyloside-Induced Apoptosis: Role of p53, ICE-Like Proteases and the Mitochondrial Permeability Transition. Journal of Cellular Biochemistry. 01 August 1997, Vol. 66, pages 245-255, especially page 245.	4, 35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/01957

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS
search terms: apoptosis, mitochondria, cell free, lysate, extract, activate, inhibit, induce, atractyloside, caspase, cpp32